University of Nottingham
UK | CHINA | MALAYSIA

Identification and Surveillance of Antibiotic Resistance in *Helicobacter pylori* in the UK

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

December 2024

Elizabeth Margaret Garvey

20004962

Supervisors: Dr Karen Robinson and Dr Neil Thomas

School of Medicine

University of Nottingham

Declaration

Unless otherwise stated, the work presented in this thesis is my own.

No part has been submitted at this, or any other institute of learning.

Elizabeth Margaret Garvey

December 2024

Acknowledgements

I would like to thank and acknowledge my principal supervisor Dr Karen Robinson for her endless kindness, support and understanding over the course of this PhD, and my co-supervisor Dr Neil Thomas for the valuable input and mentorship.

To the *Helicobacter* group for all the knowledge and joy each of you brought to my time at Nottingham, thank you all. Particularly, thank you to Jo Rhead who provided expertise, kindness and support when I needed it most, and Suffi Suffian, Farah Mahmood and Naveen Bakshi for their collaboration with the AMR testing.

Thank you to Dr Daniel Whiley, Dr Jonathan Thomas, and Dr Ibikunle Idowu for their brilliant advice and assistance with the bioinformatics analysis.

To Jen Cammarn and Alex Merritt, I cannot express how thankful and lucky I am for your support and friendship throughout the thesis writing, and to all my friends near and far who have cheered me on.

Thank you to all the members of my family, who have encouraged me and supported me in every way that a person can be supported. I will always be grateful to be part of such a mad and lovely bunch.

Finally, to my wonderful husband Jack Garvey, none of this would have been possible without you. Thank you for believing in me from the start.

Abstract

Helicobacter pylori is the leading cause of peptic ulcers and gastric cancer. Treatment is becoming more problematic due to increasing antimicrobial resistance (AMR) rates globally. There is no routine surveillance of *H. pylori* antimicrobial sensitivities in the UK, and published data is lacking as culture-based sensitivity testing is time-consuming and technically involved. This study aimed to characterise antimicrobial sensitivities of isolates collected in Nottingham since 2001, and to compare levofloxacin phenotypic and genotypic resistance rates. Additionally, whole genome sequencing methods for determining antibiotic resistance in clinical isolates were investigated, and how that data can be used with online resistance detection tools to predict phenotypic resistance.

Gastric biopsy samples were collected, with informed written consent and ethics approval, from patients attending the Queen's Medical Centre in Nottingham for an upper GI tract endoscopy. Antibiotic sensitivity was assessed using disc diffusion and E-test methods, with strain NCTC (National Collection of Type Cultures) 11637 as a control. Of 241 isolates tested, 27.8% were resistant to clarithromycin, 61.8% to metronidazole, and 2.5% to amoxicillin, which are used in first-line therapies. For those used in second-line therapies, 0% of isolates were resistant to tetracycline, and 4.1% to levofloxacin. Multi-drug resistance was found in 27.4% of isolates. Resistance to clarithromycin increased dramatically between 2001-2005 and 2011-2018 (15.6% to 40.0%; p=0.011).

Inappropriate prescription of antibiotics leads to treatment failure and the development of further resistance. In localities with high levels of clarithromycin resistance, alternatives such as levofloxacin should be considered, although use should be safeguarded to prevent the further development of resistance. Genetic determination of levofloxacin resistance in the *gyrA* gene has previously been investigated; however, genotypic sensitivity testing does not reliably predict phenotypic resistance. To investigate levofloxacin resistance mechanisms, the quinolone resistance determining region in the *gyrA* gene of 37 *H. pylori* isolates were amplified via PCR and Sanger sequenced. The minimum inhibitory concentration (MIC) and sequencing data were compared. Of the 9 phenotypically resistant isolates, 88% had a substitution at *gyrA* amino acid position 87 or 91. Every isolate containing a substation at position 91 or an Asn87Lys substitution had a resistant MIC of ≥32 to levofloxacin, evidencing that those mutations are predictors

for resistance. One phenotypically resistant isolate did not contain any *gyrA* mutations.

To further investigate this, whole genome sequencing (WGS) was undertaken. However, *H. pylori* has a highly mutable genome which makes the correct assembly of short-read sequences problematic. Conversely, the higher error rate of long-read sequencing can obscure the presence of resistance polymorphisms. Therefore, a hybrid approach was tested. 13 isolates with a range of levofloxacin MICs were selected for whole genome sequencing using Illumina and Oxford Nanopore Technologies. The long-read sequencing data were used to construct *de novo* genome assemblies. Polishing using long- and short-read data increased the genome length and mean quality while decreasing sequencing gaps. Polished genome sequences were analysed for resistance genes using the Comprehensive Antibiotic Resistance Database (CARD). The agreement rate between genotypic and phenotypic resistance to both levofloxacin and tetracycline was 100%, metronidazole was 61%, while clarithromycin and amoxicillin had low accordance with 23% and 0% respectively.

In summary, resistance rates to clarithromycin and metronidazole were higher than had previously been estimated for UK isolates. Based on the resistance profiles, treatment failure is more likely to occur when patients are given first-line therapies without amoxicillin. Levofloxacin resistance can be predicted from sequencing of the *gyrA* gene, and polishing of whole genome sequences can be used to confidently identify levofloxacin resistance genes using the online tool CARD. Based on this data, it is recommended that resistance rates of *H. pylori* should be more extensively monitored, and alternative eradication therapies should be considered. Further advancement of PCR and WGS-based AMR detection methods should be explored.

Table of Contents

List of Abbreviations

BabA	Blood Group Antigen-Binding Adhesin
BLAST	Basic Local Alignment Search Tool
CagA	Cytotoxin-associated gene A
CagPAI	Cytotoxin-associated gene Pathogenicity Island
CARD	The Comprehensive Antibiotic Resistance Database
FDA	Food and Drug Administration
i1/i2	Intermediate Region
Le Antigen	Lewis Antigen
m1/m2	Mid Region
MALT	Mucosal-Associated Lymphoid Tissue
MIC	Minimum Inhibitory Concentration
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NF-κB	Nuclear Factor kappa B
NGS	Next Generation Sequencing
NSAID	Non-Steroidal Anti-Inflammatory Drug
OMP	Outer Membrane Protein
ONT	Oxford Nanopore Technologies
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
PUD	Peptic Ulcer Disease
QRDR	Quinolone Resistance Determining Region
RGI	Resistance Gene Identifier
s1/s2	Signal Region
SabA	Sialic Acid-Binding Adhesin
SAT	Stool Antigen Test
SNP	Single Nucleotide Polymorphism
T4SS	Type IV secretion system
TLR	Toll-Like Receptor
UBT	Urea Breath Test
UKHSA	UK Health Security Agency
VacA	Vacuolating Cytotoxin A
WGS	Whole Genome Sequencing
WHO	World Health Organization

List of Figures

List of Tables

Chapter 1: Introduction

1.1 *Helicobacter pylori* Infection and Disease

1.1.1 General Overview

Helicobacter pylori is a widespread human pathogen, present within the stomachs of nearly half of the world's population. Once established, these Gram-negative, spiral-shaped bacteria will usually persist for the duration of a person's life if no effective treatment is administered [1]. Although the majority of infections are asymptomatic, *H. pylori* can cause chronic inflammation of the stomach mucosa, which may develop into gastric or duodenal ulcer disease in 10-20% of cases. It is also one of the leading causes of gastric cancer and is the fourth most common cause of cancer-linked death [2]. As such, the World Health Organization (WHO) International Agency for Research on Cancer (IARC) has designated *H. pylori* as a biological carcinogen [3] and clarithromycin-resistant *H. pylori* had previously been listed as a WHO class 1 priority pathogen for drug development [4].

Public Health England (now called the UK Health Security Agency – UKHSA) published guidelines in 2019 recommending that antimicrobial susceptibility testing should be performed when a

patient has undergone two failed rounds of eradication therapy [5]. However, this is often not carried out as the organism is difficult to isolate and culture, and as such there is little local resistance data available for clinicians to consult when considering the most effective treatment options. Antibiotic therapy is currently still the only viable established method for tackling this infection, although increasing rates of antibiotic resistance are jeopardising their effectiveness. This highlights the vital need for up-to-date and relevant antibiotic resistance data and for further understanding of antibiotic resistance mechanisms in *H. pylori*.

1.1.2 History and Discovery of *Helicobacter pylori*

The link between *H. pylori* and gastritis was not known for many years, and although there had previously been efforts to identify if bacteria were the causal agent of stomach ulcers [6], *H. pylori* itself was not cultured until 1982, with the results published in *The Lancet* in 1984 [7]. For a long time, it was thought that gastric ulcers formed because of stress and dietary factors. It was widely assumed that because of the highly acidic nature of the stomach, it was a sterile region where bacteria could not survive [8]. In 1979, pathologist Robin Warren noticed bacteria adhering tightly to epithelial tissue that had been taken from a patient with severe

gastritis. This led to an investigation that spanned the next 18 months. Warren focussed on biopsy samples from patients with and without gastritis, determining that the bacteria were rarely observed in tissue sections from the stomachs of healthy patients and were instead closely associated with the samples from gastritis patients [7].

Warren collaborated with Barry Marshall at Royal Perth Hospital to further research the bacteria. Culturing *H. pylori* from gastric tissue proved extremely difficult at first, until one Easter weekend where a set of culture plates were accidentally left to incubate for several days, and small clear colonies were observed on return to the laboratory. After attempts at inoculating pigs to induce gastric disease failed, Marshall decided to infect himself by ingesting a sample of his culture stock. After a week, he developed symptoms of vomiting and nausea, and a biopsy confirmed that he had developed gastritis, from which the bacteria could be cultured. He took a treatment of metronidazole and bismuth salts, after which the symptoms abated and further biopsies returned clear, thus confirming Koch's postulate, and providing further evidence for a causal link between the bacteria and the disease [9]. This discovery was provisionally identified as a *Campylobacter*-like bacterium, however, genetic testing later confirmed that they were

not of the same genus as other *Campylobacter* species. As such, the name *Helicobacter* was chosen for the new genus based on the spiral morphology of the bacteria, shown in **Figure 1** below [10]. Their research identifying that gastritis was an infectious disease caused by *H. pylori* led to Warren and Marshall winning the Nobel Prize in Physiology or Medicine in 2005 [11].

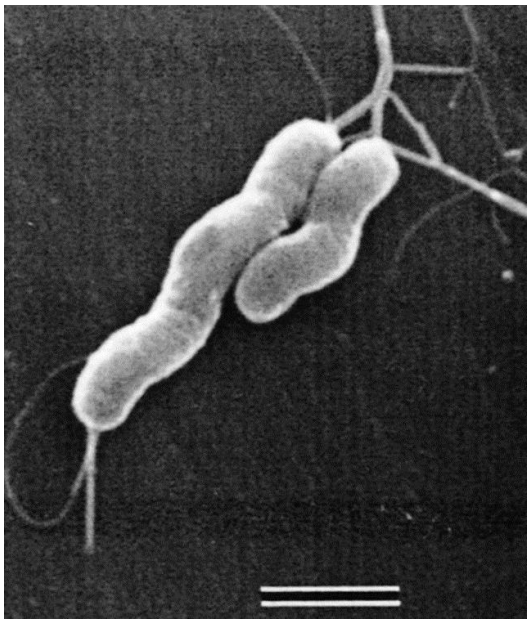


Figure 1 *- An electron micrograph image of H. pylori.*

The spiral morphology and polar flagella are visible. Scale bar = 1 μm. Image taken from Yoshiyama & Nakazawa (2000) [12]

1.2 *H. pylori* and Disease

1.2.1 General Overview

The circumstances of why some *H. pylori* infections result in disease are not fully understood yet, although host genetics and environmental factors such as smoking and diet have been shown

to contribute [13]. *H. pylori* virulence factors also play a role, particularly the presence of the cytotoxin-associated gene pathogenicity island (*cag*PAI), which is a major risk factor for the development of disease and cancer, due to the encoded cytotoxin-associated gene A (CagA) protein which induces inflammatory signalling in host epithelial cells [14]. Additionally, expression of the more active s1/i1 form of the vacuolating cytotoxin A (VacA), named for its ability to induce the formation of vacuoles within gastric epithelial cells, has been linked to the development of disease [15].

Eradication of *H. pylori* is the foremost recommended treatment for peptic ulcer disease (PUD), and immediate eradication is recommended as a preventative measure against the progression of gastric mucosal-associated lymphoid tissue (MALT) lymphoma. In the UK, treatment usually includes a triple therapy of drugs, consisting of two antibiotics and a proton pump inhibitor (PPI) such as omeprazole to suppress the secretion of gastric acid. Typically, the antibiotics prescribed will include clarithromycin, and either amoxicillin or metronidazole [16]. Treatment and diagnosis guidelines for *H. pylori* emphasise the importance of determining patient history of antibiotic use, and consideration of local antibiotic resistance rates when deciding on an appropriate

treatment regimen, as these are both factors that can negatively impact the likelihood of treatment success [17]. Currently, susceptibility testing relies on culturing *H. pylori* isolated from the patient's stomach, via endoscopic gastric biopsy collection. Biopsies can be uncomfortable and expensive procedures that require a hospital appointment, which is a contributing factor to the lack of routine culturing and screening of *H. pylori* isolates in the UK.

1.2.2 Transmission

H. pylori is closely associated with its human host population. The most likely modes of transmission of the bacteria to new hosts are the gastro-oral, faecal-oral, and oral-oral routes, however exact mechanisms have not yet been identified [18]. The main site of infection is the human stomach, although *H. pylori* has also been identified as present within saliva and dental plaque [19], particularly after vomiting, regurgitation, or gastro-oesophageal reflux. Contaminated saliva can therefore act as a means of oral-oral transmission. It is thought that the infection is usually acquired during childhood, as the lower gastric acid output allows a bacterial population to survive in the gastric juice, which allows time for the bacteria to migrate into the protective mucus layer of the stomach and become established. Once there, untreated infections usually persist life-long within the stomach, most commonly asymptomatically [20]. *H. pylori* is predominantly

spread between family members [21] evidenced by the occurrence of clusters of genetically related isolates within family groups [22]. It is mostly transmitted from parent to child, although it has also been observed to spread between siblings [23]. Socioeconomic factors including poor hygiene and sanitation have also been linked to increased incidence of infection [24].

Dietary factors also have an impact on transmission. It has been suggested that high salt [25] and coffee consumption may be linked to an increased risk of *H. pylori* infection, while alcohol consumption may have a protective effect [25], [26], although alcohol use has also been linked to treatment failure [27]. While it is possible to isolate *H. pylori* from domestic animal hosts such as cats and dogs, there is little significant evidence to suggest that transmission from animals to humans is a major form of disease spread. It is likely that instead, transmission to domestic cats occurs from human hosts, as *H. pylori* incidence in feral cat populations living away from humans is low [28]. However, there is some evidence that transmission from livestock like sheep and cattle may be possible in people who regularly interact with infected livestock faeces and milk products, again most likely via the gastro-oral route [29], [30].

1.2.3 Colonisation

1.2.3.1 Urease Production

H. pylori is uniquely equipped for settling and surviving within the gastric mucosal niche. However, successful colonisation of a new individual depends on short-term exposure to conditions both outside the human host, and within the acidic stomach lumen. To facilitate this journey, *H. pylori* has developed a number of survival adaptations, particularly gastric adhesins, motility functions, and acid neutralisation in the form of urease production. The pH of gastric juices within the human stomach is generally within the range of 1.5-2 [31] which is usually acidic enough to kill bacteria within a 15-minute timeframe [32]. *H. pylori* has evolved so that in acidic (H^+) environments, it will produce the enzyme urease. In the bacterial periplasm, the urease enzymes convert urea from the host tissue fluid into ammonia ions, which neutralises the hydrochloric acid in the bacteria's local environment and provides a pH buffer. *H. pylori* produces more urease than any other protein, with the enzyme accounting for 10-15% of its total protein production by weight [33]. High levels of urease production are an essential factor for both initial and continued colonisation, as evidenced by animal models. Experiments were conducted where gerbils were infected with a strain of *H. pylori* that had an inactive version of the gene responsible for urease production (*UreI*), and said strains were unable to maintain colonisation [34]. As such, the

elevated presence of urease enzymatic activity in the stomach associated with *H. pylori* infection has led to a useful means for detection and diagnosis of infection in the form of urease breath tests [35], discussed further below.

1.2.3.2 Adhesion

Another major factor of *H. pylori* colonisation is adhesion. Glycoproteins that form a large component of the mucus layer of the stomach provide surfaces that antigens on the *H. pylori* cell exterior can bind to. The *H. pylori* bacteria therefore tend to aggregate in these areas of the stomach and can persist there even during gastric emptying. MUC5AC and MUC6 are large gel-like glycoproteins that are produced as part of the stomach's protective mucus, and both play a large role in *H. pylori* adhesion [36]. There are zones of differential expression within the stomach, with surface epithelial cells usually expressing the mucin MUC5AC, and the deeper gastric glands expressing MUC6. MUC2 is usually only expressed when gastric tissue undergoes intestinal metaplasia, which in some cases can lead to cancer. The presence of MUC2 is therefore an indicator of disease progression [37].

The gel-forming MUC5AC mucin displays a particular carbohydrate structure, called a Lewis (Le) antigen on its surface. Lewis antigens

are also expressed on red blood cells, and so are referred to as blood-group antigens. Subsets of these Lewis antigens, particularly the Lewis B antigen (Le^B) have been shown to have high binding affinity to the *H. pylori* surface protein 'blood group antigen-binding adhesin' called BabA [38]. BabA has additionally been found to have affinity for a large array of host stomach antigens, such as MUC1 and Globbo A & H, as well as MUC5B and MUC7 found in saliva [39].

Another *H. pylori* outer membrane protein, (SabA) can bind to host cell glycans that have had sialic acid covalently bound to them, particularly Lewis X (Le^X) antigen. This sialyl Le^X is expressed on inflamed gastric epithelial cells, which is a symptom of chronic *H. pylori* infections, as shown in **Figure 2** below. Taken together, BabA binding is important for initial anchoring and establishment of infection, while SabA binding is useful for persistence of infection through host inflammation [39]. An *in vitro* study showed evidence that increased concentrations of salt also upregulate the expression of SabA, along with other outer membrane proteins and virulence factors like VacA in *H. pylori* [40], which could be a factor in why a high salt diet is a risk factor for gastric cancer [41].

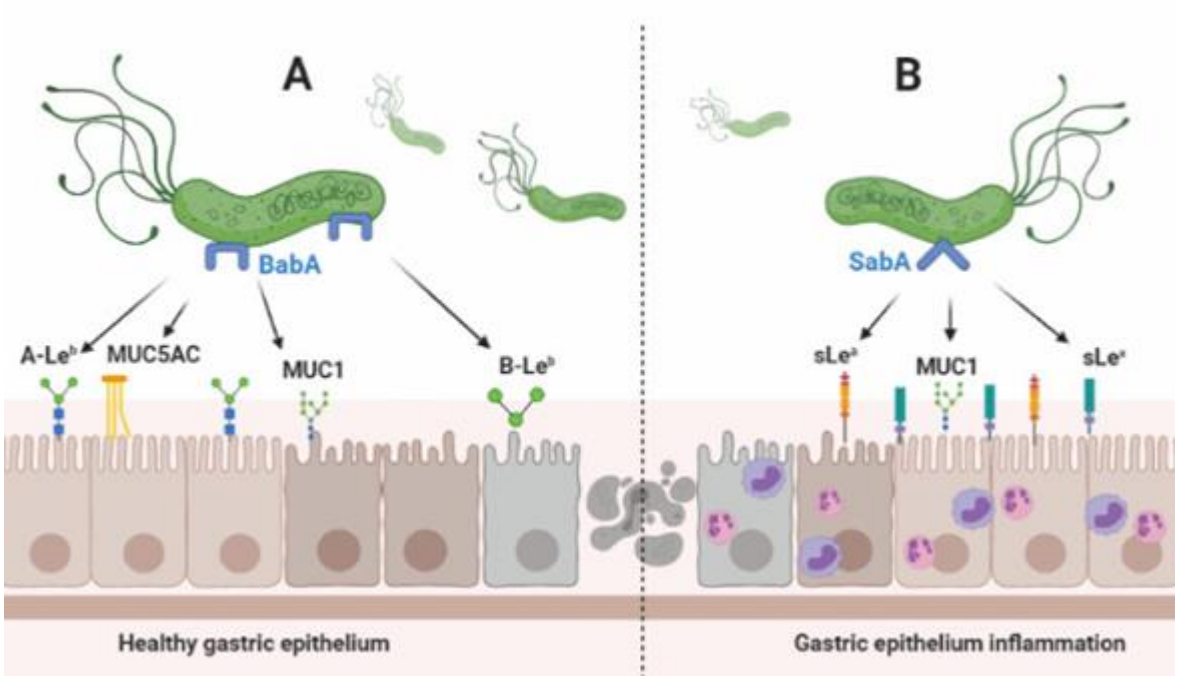


Figure 2 *- The process of H. pylori adhesion in healthy and inflamed gastric epithelial tissue.*

H. pylori expresses surface proteins, which facilitate bacterial binding to host antigens. A) BabA expressed by H. pylori exhibits high binding affinity to antigens expressed in healthy epithelial tissue, such as MUC5AC, MUC1, and Lewis blood group antigens Le^A and Le^B. B) H. pylori protein SabA binds to host antigens that have had sialic acid covalently bound to them, which are more often expressed in inflamed tissue e.g. sialyl Lewis A and X antigens. Image taken from Doohan et al., (2021) [39].

1.2.3.3 Structure and Motility

Another essential feature of *H. pylori* infection is motility, provided by the flagella concentrated at one pole of each bacterium. Experiments that have been able to construct non-motile *H. pylori* have shown that rate of infection in mouse models is significantly reduced compared to the wild-type control [42], and in a piglet model, the motile form survived 21 days compared to just 6 days in the non-motile form [43]. The flagella of *H. pylori* are similar to those in *Campylobacter* species and consist of between two and six

long threads at one pole of the bacteria. These filamentous constructs are composed of structural proteins, a protective sheath that is an extension of the outer membrane of the bacteria, and a terminal bulb [44]. Each one extends around 3-5μm from the end of the bacterium. Changes to both the structure and the functionality of these flagella have both been found to impact colonisation capabilities [45]. The spiral corkscrew shape of *H. pylori* plays a role in helping the bacteria burrow into the mucus layer, as mutants that have an altered cell shape have a reduced colonisation capacity despite being equally motile compared to wild-type strains [46]. Chemotaxis is another element that plays a role in *H. pylori* migration, guiding the bacteria to the mucosal layers with the most appropriate pH for survival [47].

The majority of *H. pylori* in an infected stomach are found free-swimming within the mucus layer, with a smaller proportion found close to the gastric epithelial cell surface. While this mucosal layer acts to protect *H. pylori* bacteria somewhat from the acid and digestive enzymes of the stomach lumen, it also undergoes a constant process of sloughing off and being replaced by mucosal secretions from gastric glands – a method by which the host prevents bacterial colonisation and protects the stomach epithelial cells from being damaged by the acid. However, *H. pylori* has been

shown to reduce the rate of mucin turnover within the stomach, which allows it to settle more securely into the stomach environment without being removed by the physical defences of the body [48]. Additionally, *H. pylori* raises the pH of the environment surrounding it with urease enzymes, which also makes the mucus in contact with the bacteria less viscous and easier to transverse [45]. It can also produce a protein called thioredoxin reductase, and it has been postulated that this may work to disrupt disulphide bonds within the mucin layer and weaken it [49].

1.2.3.4 Colonisation and Niche Diversity in the Antrum and Corpus of the Stomach

Several parts of the stomach can be colonised by *H. pylori*, with the corpus (body) and antrum being of the most relevant. The environments of these two regions vary greatly in some respects such as pH and thickness of mucosa, providing two diverse niches. In a healthy stomach, the corpus contains glands with a higher number of parietal cells, which are responsible for secreting hydrochloric acid, a major component of stomach acid. They also contain gastric chief cells, involved in the production of digestive enzymes such as lipases and pepsins [50], and tend to be deeper than antral glands, as shown in **Figure 3** below. The antrum,

however, has glands that mostly contain mucus producing cells and endocrine cells that secrete acid regulating hormones. The two major endocrine cells are called D cells (which produce somatostatin) and G cells (which produce gastrin). Somatostatin and gastrin work in concert with each other, with gastrin from the bloodstream acting on the corpus glands to increase gastric motility, gastric acid secretion, and mucosal production in the stomach. Somatostatin from the D cells in turn acts as a regulator for the G cells, supressing gastrin production. The antrum has a thicker mucosal lining than the corpus, and is also generally less acidic, with an average pH of 4.4 compared to a pH of 3.7 in the corpus [51].

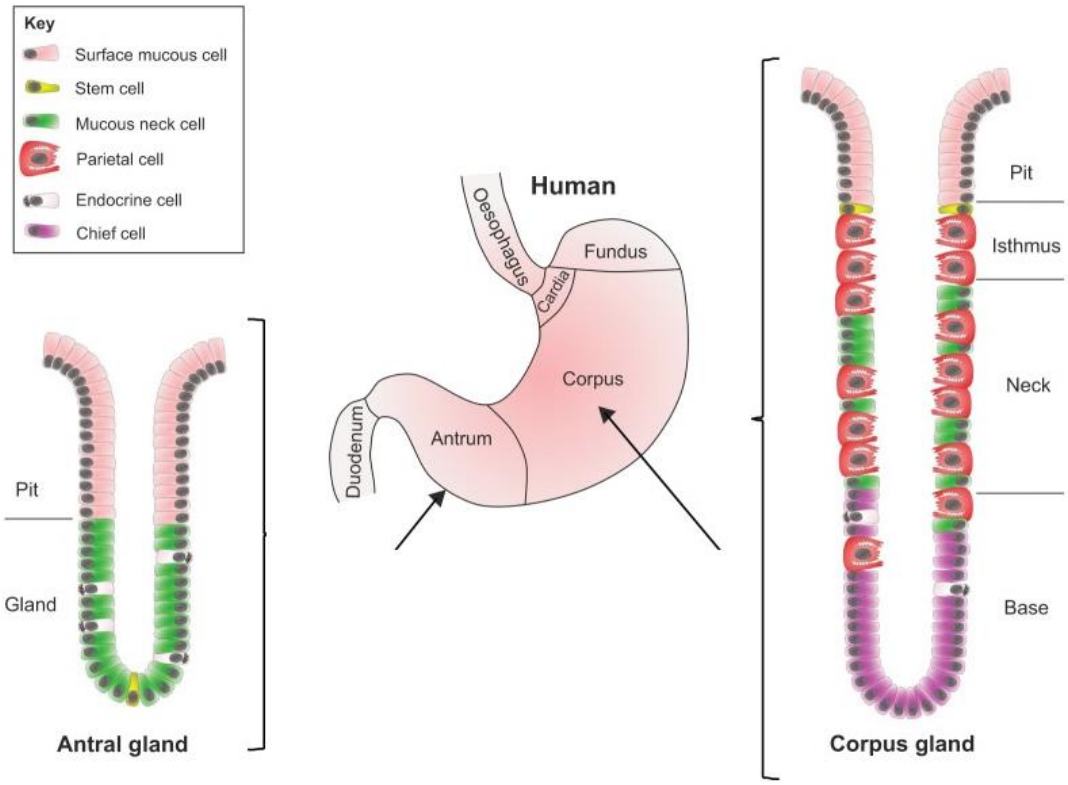


Figure 3 - *The anatomy of the human stomach, and structure of the antral and corpus glands.*

The glands present in the antrum and corpus of the stomach contain different cell types. Due to the different structure and function of the glands, there are different environmental niches within the areas of the stomach. Antral glands mostly consist of the mucus producing neck cells and surface mucus cells, with endocrine cells that produce the hormones gastrin and somatostatin. Gastrin acts on cells within the gland to increase production of acid and mucus, while somatostatin acts on the endocrine cells to reduce gastrin production. The parietal cells produce hydrochloric acid and are only present in the deeper corpus glands. Chief cells within the base of the corpus glands produce digestive enzymes such as pepsin and chymosin. Stem cells are present within both types of gland, which proliferate and differentiate into new cells to replace any lost. Image adapted from Burkitt & Duckworth et al., (2017) [50].

1.2.3.5 Disease Patterns from Infection in the Antrum and Corpus of the Stomach

When colonising the stomach, *H. pylori* can form population pockets within the gastric pits of the antrum and corpus, because of the relatively stable environment provided there. Infection of the glands in the antrum compared to the corpus can lead to distinct disease outcomes, because inflammation impacts the functionality of the glands and causes different effects on the stomach, as shown in **Figure 4**. Proteomics performed on animal models has shown that infected antrum and corpus tissue displays noticeable changes in protein expression in comparison to healthy stomach tissue, including the suppression of transcription factors for hormones that modulate acid production [52].

Briefly, inflamed antral glands lead to increased stomach acid production, and can cause the development of duodenal ulcers. Conversely, inflammation in the corpus glands of the stomach leads to reduced acid output. With reduced acidity, *H. pylori* and other bacteria can more freely colonise the gastric glands and lead to even more inflammation. Chronic inflammation of the glands can cause them to atrophy, and in some cases can develop into gastric adenocarcinoma. The site of initial inflammation leads to either an increase or decrease of stomach acid production, which means that

the different disease outcomes caused by the different acid levels rarely occur simultaneously within an individual [53].

The mechanisms of this are as follows. In the event of *H. pylori* colonisation and inflammation within the antrum (antral-predominant gastritis), the normal function of the antral glands is altered. The endocrine cells within the antral glands secrete higher levels of the hormone gastrin, causing the parietal cells to increase levels of gastric acid production. Somatostatin release is also suppressed, which usually acts to limit gastrin production, leading to a further increase in gastric acidity. Low pH can cause damage to the duodenum – the first part of the small intestine immediately following the stomach. The DNA damage to duodenal cells caused by this acidity can lead to gastric metaplasia, a process which involves the replacement of duodenal epithelial cells with epithelial cells normally found in the stomach. Gastric-type cells in the epithelium can then be colonised by *H. pylori* and may lead to duodenal ulceration [1].

If *H. pylori* colonisation is concentrated in the corpus however, pro-inflammatory factors can instead cause the suppression of gastric acid by inhibiting the acid output of parietal cells [1]. An increased pH leaves the stomach vulnerable to colonisation from other

microbes and can cause atrophy and loss of the gastric glands. Eventually, this increases the risk of developing gastric ulcers and adenocarcinomas, which are masses of cell growth within the mucus-producing cells of the stomach. These tumours may require surgery to remove, and unfortunately once established have a poor survival rate due to symptoms often not being noticeable until the disease is well advanced [54].

Colonisation may also occur in both areas of the stomach simultaneously, either with homogenous strains or distinct strains that coexist within the two niches at the same time. These dichotomy in these strains may be due to having fitness advantaged specialised for the separate niches, and some have been found to display differences in the expression of virulence factors and patterns of antimicrobial resistance [55]. Sequencing of diverse strains from individual patients showed phylogenetic clustering, suggesting that in some cases patients may be infected with an individual strain that then genetically diverged [56], possibly because of the different selection pressures of each niche. Additionally, people can be infected by two or more distinct strains at once [57]. The ramifications of these multi-strain infections are that a biopsy sample taken from only one site of the stomach may not provide a full picture of the attributes of the infection.

Antimicrobial resistance profiles and virulence types of the infecting strains in other areas of the stomach may differ, and lead to ineffective treatment regimens [58]. A potential outcome of this may be that *H. pylori* is eradicated in one area of the stomach but not the other, leading to recolonisation of the stomach and re-emergence of infection [59].

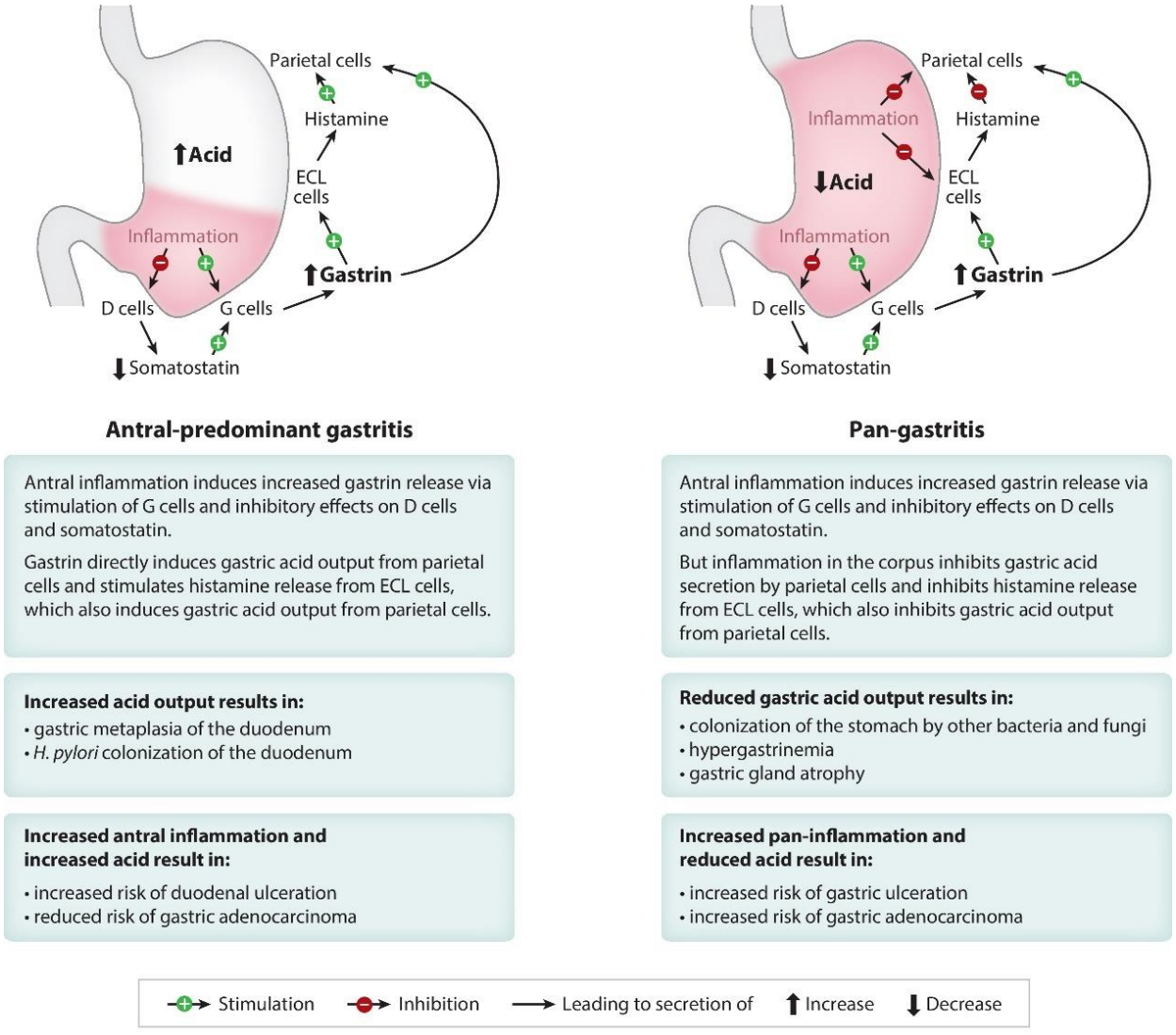


Figure 4 *- The effect of the site of inflammation on gastric acid production and disease outcome in human stomachs.*

Inflammation concentrated in the antrum is associated with increased risk of duodenal ulceration, compared to infection of the corpus which is associated with increased risk of gastric adenocarcinoma. ECL - enterochromaffin-like cells, which release the immune transmitter histamine. Image taken from Robinson & Atherton, (2021) [1].

1.2.3.6 Disease Manifestations

The clinical outcome of *H. pylori* colonisation can vary greatly between patients, ranging from being completely asymptomatic to severe disease depending on several factors. Bacterial strain virulence, host genetics and the nature of the immune response, and environmental factors such as high salt diet and smoking are all thought to influence disease risk. Disease outcomes can include peptic ulcer disease and gastric cancer, which is the fourth most common cause of cancer death worldwide [60]. *H. pylori* induced inflammation is the strongest disease risk factor for gastric cancer, and as such the pathogen is categorised as a Class I Human Carcinogen [61], [62]. The vast majority of people colonised with *H. pylori* will remain asymptomatic for the duration of the infection, however over the course of a lifetime, it has been estimated that 10 - 20% of infected people will develop a form of ulcer disease, and around 1-2% develop some form of gastric cancer [21], [63], specifically MALT lymphoma, or more commonly gastric adenocarcinoma [64]. The 5-year survival rates for those with gastric cancer in the UK is 19%, which may be due to many cases already being metastatic by the time of diagnosis. Additionally,

patients tend to be older (aged 70-75) when diagnosed, and treatment options are limited [64]. Eradication of *H. pylori* is the first line treatment for early-stage MALT lymphoma patients, with disease regression observed in 75% of patients treated in this way [65].

As previously discussed, *H. pylori* is also one of the primary causes of PUD, where ulcers can develop in the body of the stomach (gastric ulcers) or within the duodenum (duodenal ulcers) and may lead to life-threatening complications such as bleeding and perforation. Ulceration usually occurs when the defensive mucosal lining of the stomach is compromised, allowing for infiltration by stomach acid and digestive enzymes, causing inflammation and damage to the epithelial cells [66], [67]. The progression of the disease from colonisation to cancer is largely due to chronic inflammation at the site of infection, which can cause the production of DNA damaging reactive oxygen species. Some strains of *H. pylori* are also more pathogenic than others, such as those expressing the virulence factors *cagA* and more active forms of *vacA* such as *vacA* i1 [68]. These factors stimulate inflammatory signalling in host cells, alter epithelial cell morphology, disrupt tight junctions, and increase the risk of carcinogenesis [61], [62], [69]. These virulence factors will be discussed in further detail below.

Host immune factors also impact disease progression of *H. pylori* infection. A chronic inflammatory response without clearance of the bacteria is a major risk factor for carcinogenesis [70]. Several host immune response gene polymorphisms have been reported to influence disease progression. Toll-like receptors (TLRs) are molecules expressed in the gastric epithelium, and when activated by the binding of pathogen-associated ligands cause a pro-inflammatory host response. TLR binding leads to the activation of transcription factor nuclear factor kappa B (NF-κB), which causes expression of inflammatory cytokines [71]. Variations in the genes that encode some types of TLRs can lead to increased immune activation and inflammation in some individuals, which is linked to more severe gastritis and may increase the risk of cancer development [72]. Smoking and usage of non-steroidal anti-inflammatory drug (NSAID) painkillers can also contribute to the development of ulcer formation and disease. Dietary habits such as high levels of salt consumption are a disease risk factor, both in terms of encouraging inflammation and upregulating VacA production by the bacteria [40].

1.2.3.7 Reinfection

After successful eradication treatment for *H. pylori*, recurrence of the infection in adults is uncommon, largely because gastric acids and protease enzymes within the adult stomach provides protection against recolonisation. However, it is still a possible for

recolonisation to occur due to reservoirs of infection both within the body and from external sources.

A meta-analysis by Hu *et al*. of global reinfection rates of *H. pylori* pooled data from 132 studies covering 45 countries. The review looked at both the recurrence of the same infection in a patient after a failed eradication of *H. pylori* (recrudescence), and the acquisition of a new infection after a confirmed eradication (reinfection). The global rate of recrudescence was 2.2%, and the reinfection rate was 3.1%, both of which were significantly linked to poor sanitation and health standards in the local areas [73]. Areas of the mouth such as the biofilm on the tongue and the dental pulp (particularly in children) may act as secondary bacterial reservoirs [74], however it is unclear if these populations are transient regurgitated bacteria from the stomach infection, or an established site of colonisation that can act to reinfect the stomach. While in some cases it was found that the two populations can be homologous [75], other studies found there is no apparent relationship between the two groups [76].

One difficulty with confirming whether the mouth can act as a reservoir for infection is that culturing *H. pylori* from this niche is problematic and has only been achieved sporadically [77]. It has been suggested that this difficulty arises because oral *H. pylori* bacteria often transition into an inert coccoid form with low

metabolic activity, to minimise damage from oxygen and temperature changes in the mouth [78] and thus are difficult to culture with the standard techniques and conditions used. Disease recurrence is a problem not just because of the potential for further disease, but also because it can lead to the development of antibiotic resistance in some cases [28]. It is therefore important that rates of reinfection and recrudescence be minimised wherever possible.

1.3 Diagnosis

There is a range of methods for diagnosing a *H. pylori* infection, which are broadly organised into 'invasive' and 'non-invasive' categories. The primary care guidelines published by UKHSA [5] recommend testing for the presence of *H. pylori* in patients with dyspepsia, or patients with a history of duodenal ulceration/bleeding that have not previously been tested. Testing can be performed using either a urea breath test (UBT), or a stool antigen test (SAT) in people who are at a lower risk of gastric cancer (e.g. younger patients). Both are non-invasive tests that a GP can refer a patient for, and the samples are sent for analysis at a laboratory. In patients who are over 55 years of age and have a recent onset of persistent dyspepsia, an endoscopy is advised. An endoscopy is useful in these cases so that potential ulceration or

gastric cancer development can be identified, and treatment can be started as soon as possible [79]. However, endoscopies are an invasive procedure that have their own risks and require an appointment with a specialist at a hospital. UKHSA recommends a 'test and treat' protocol, suggesting that eradication therapy should be administered for every patient who tests positively for *H. pylori* [5].

1.3.1 Non-Invasive Tests

1.3.1.1 Urea Breath Test

The urea breath test is a non-invasive and highly accurate test with a specificity and sensitivity of over 98%. A high sensitivity means that there is a high probability of a positive diagnosis being correct (few false negatives), and high specificity means that there is a high probability of a negative diagnosis being correct (few false positives). This makes the urea breath test extremely useful for primary diagnostic purposes [80]. This test works because compared to other microbes, *H. pylori* produces high amounts of the enzyme urease, which hydrolyses urea into ammonia in order to create a less acidic local environment. The activity of this enzyme can be used as an indicator to identify the presence of a *H. pylori* infection, as carbon dioxide is also produced as part of this reaction [81]. Urea usually contains only 1% of the isotope ^{13}C, with the other 99% being isotope ^{12}C. The urea breath test works

by taking a baseline breath sample, and then providing the patient with a ^{13}C-urea drink or capsule to consume. After 30 minutes, another breath sample is taken. If *H. pylori* is present in the stomach, there will be elevated levels of CO_2 containing the ^{13}C isotope in the patient's second breath sample compared to the first [82]. UBTs are available with referral from a GP, and the process of providing the breath samples is short, with extremely minimal discomfort or risk for the patient. However, recent antibiotic use can depress bacterial enzymatic activity, and lead to false negatives for up to four weeks after treatment even if there is still infection present. This limits the urea breath test's capacity for use in verifying eradication immediately following antibiotic therapy. Additionally, the UBT will only give information as to whether a *H. pylori* infection is present in the stomach and gives no further diagnostic information such as disease status or antimicrobial resistance profiles [83].

1.3.1.2 Stool Antigen Test

Culturing of *H. pylori* is not usually possible from patients' stool samples as the bacteria are destroyed by bile salts and digestive enzymes within the intestines. As an alternative, *H. pylori* antigens may be detectable within faecal samples, and this forms the basis of the non-invasive stool antigen test. This test works by taking a pea sized amount of faecal matter from a patient, which is then sent for analysis in a laboratory. Antibodies are then used to detect

the presence of *H. pylori* antigens present. There are a range of SAT kits available from different manufacturers, which use different antigens and detection assays.

The Enzyme-Linked Immunosorbent Assay (ELISA) based test varies depending on the manufacturer's instructions. However, in general, the ELISA test relies on a coating of antibodies being applied to the well of a plate. These anti-*H. pylori* antibodies will capture *H. pylori* antigens present within the prepared stool sample, and the well is then washed to remove any free antibodies or antigens not captured. Depending on the kit used, another antibody is then added which binds to the captured antigen. This second antibody is labelled with a marker that can be detected e.g. by changing the colour of the solution within the well to allow identification of *H. pylori* positive samples [84]. Tests have been developed that have only one type of antibody coating the well (monoclonal) or have several types (polyclonal), which target multiple antigens such as the *H. pylori* flagella or catalase [85]. Monoclonal based kits were found to have over 96% sensitivity and specificity, whereas polyclonal kits were found to be less accurate, with 88% and 93% specificity and sensitivity, respectively [86].

An immunochromatography version of the SAT is also available, which works in a similar manner to a COVID lateral flow test, where a small faecal sample is added to a tube of buffer and shaken. This is loaded onto a flow cell. A C (control) line will appear to indicate that the test is working correctly, and a second T (test) line indicates that the sample is positive for *H. pylori* [87]. This provides rapid results without the need for specialist laboratory equipment; however, it is not as reliable as ELISA based assays [88]. The stool antigen test has been found to be useful for assessing if eradication therapy has been unsuccessful 7 days after the completion of antibiotic therapy [89]. It has also been found to perform well for diagnosis in children and other individuals for whom endoscopy is problematic [90], particularly as collecting samples for the UBT from very young children and infants is difficult due to needing them to cooperate when gathering breath samples [86]. However, SATs are also susceptible to an increased rate of false negative results due to low bacterial load caused by recent antimicrobial use before testing [91].

A stool-based real-time polymerase chain reaction (PCR) test has also been developed to detect clarithromycin-resistant *H. pylori* non-invasively, by identifying mutations within the *H. pylori* 23S rRNA gene [92]. An earlier 2016 evaluation of the commercial

Geno Type HelicoDR molecular test had compared biopsy and stool samples for detection of clarithromycin and had found only a 53% rate of agreement between the two [93]. However, a more recent meta-analysis of stool-based PCR tests has found high levels of diagnostic accuracy, with 91% of samples tested correctly detecting markers for clarithromycin resistance [94]. Another variant of the test called the Nested PCR-quenching probe method demonstrated high consistency with resistance results from conventional culture-based susceptibility testing, with 21 out of 21 tests returning concordant results [92]. This same study demonstrated that clarithromycin-based triple therapy was more likely to fail in patients who had tested positive for *H. pylori* strains with common clarithromycin resistance mutations, and as such stool-based PCR testing could be beneficial for tailoring antibiotic options to avoid treatment failure. In the future, these non-invasive tests could be expanded upon to allow identification of other types of AMR genes too, such as detecting mutations in the *rdxA* gene that can confer resistance to metronidazole [94].

1.3.1.3 Serology

Serology is classed as a non-invasive test that detects the presence of *H. pylori* IgG antibodies in a patient's blood sample. Several methods can be used to identify if antibodies are present in

the sample, including agglutination, Western blotting, immunochromatography, and ELISA. Taking a blood sample can be performed by a local GP, and then analysis of the sample can be done either in-house or by sending the sample to a laboratory and is a low-cost test to run [83]. It should be noted, however, that because serology is used to detect IgG antibodies, it cannot be used to determine if an infection is still ongoing or has been cleared in the past. This would require a detectable fall in serum antibody titre, which may take more than a year [95]. Serological testing is in general not as accurate as the other non-invasive tests; however, it can be useful in determining infection in patients with a GI bleed, which can cause false negative results in UBT or stool antigen tests [5]. There are commercially available laboratory ELISA kits that have a high sensitivity (90-100%) but a specificity that can be as low as 76%. It is possible, however, to identify *cagA* and *vacA* virulence factor status with the use of certain serological tests [95]. These are not currently routinely used in clinical diagnostics, although some recent studies have found that there could be some use for them in the future, particularly in populations with high local levels of gastric cancer [96].

1.3.2 Invasive Tests

1.3.2.1 Endoscopy

Biopsy samples taken during a gastric endoscopy can be used to isolate and culture *H. pylori*, and thus confirm the presence of infection. Features of the stomach tissue that are visible during the endoscopy such as inflammation and ulceration can also help to inform a diagnosis, but not with more accuracy than any of the non-invasive tests [83]. Performance of this procedure requires a hospital visit with a trained specialist, which may mean that a patient needs to wait for an available appointment, which can delay diagnosis. There is also a certain amount of risk of bleeding and perforation of the stomach involved with the procedure, and discomfort to the patient when performing a biopsy of the stomach [97]. An endoscopy can provide important diagnostic data however, such as detection of diseases related to *H. pylori* infection, including ulcers, gastritis, and gastric cancer. Biopsy samples taken from the stomach can also be used for histology, and for culturing *H. pylori* isolates. Cultured isolates provide more information than the non-invasive tests, as they can be used to determine an antibiotic resistance profile of the infection as well as the *vacA* and *cagA* virulence factor status of the strain [98]. This is not performed routinely however, and the isolation and culturing of

the bacteria can take many weeks. Many clinical labs are also not equipped for culturing *H. pylori*, which is fastidious and slow growing.

1.3.2.2 PCR

PCR of DNA extracted from biopsy samples can be used to identify both the presence of *H. pylori* DNA, and the detection of virulence factors such as *vacA* and *cagA* [83]. Additionally, information about the potential antibiotic sensitivity profiles of the bacteria can be collected by amplifying genes such as the 23S rRNA and *gyrA* genes, some mutations of which may be linked to clarithromycin and levofloxacin resistance respectively [99]. This will be discussed further in Chapter 4.

The above diagnostic methods all have positive and negative aspects which can affect the suitability of each one for use, depending on the needs of the patient. Globally, they are also dependent on what local expertise and laboratory equipment are readily available, and so different tests will likely be appropriate in different settings [100]. In the UK, guidelines from the UK Health Security Agency specify that the UBT and SAT are used in cases of uncomplicated dyspepsia, and serology is not recommended [5].

1.4 Identifying Resistance

Antimicrobial resistance in *H. pylori* can be identified either phenotypically or genotypically, using culturing techniques or DNA testing, to make a profile of which drugs are more likely to be effective in a treatment plan. Both methods have advantages and drawbacks, which will be discussed in greater detail in later chapters. Although it is recommended by UKHSA that antibiotic susceptibility testing is performed after two failed rounds of eradication therapy, this is often not carried out and data about local resistance rates is scarce.

1.4.1 Disease Prevalence

The prevalence of *H. pylori* infection and disease varies by location, and is mainly influenced by virulence factor expression, but also smoking status, age, and socioeconomic factors. Worldwide, the continent that has the highest infection rates is Africa, with a pooled estimated resistance rate of 70% [101]. This can vary within regions, with Nigeria having a *H. pylori* infection prevalence of 88% [102], whereas adults in Kenya have a reported rate of 55% [103]. The region of lowest prevalence rate was reported to be Oceana at 24%, however, in Australia, rural Indigenous populations had a far higher infection rate (75%) than the general population (25%). The country with the lowest *H. pylori* infection

prevalence is Switzerland at 20% [101]. It should be noted that these data are informed estimations, and the true infection rates may be different between research groups based on testing methods and sampling biases.

1.4.2 Demographics

1.4.2.1 Disease and Infection Demographics

Infection prevalence does not always directly reflect disease likelihood. Some areas of the world have proportionally higher disease prevalence compared to infection rates. Strains containing more active forms of the *cagA* and *vacA* genes are more common in different localities around the world. For example, Africa has one of the highest infection rates in the world, however, gastric cancer rates remain low in some countries, possibly due to an absence of the *cag*PAI in the local isolates [104]. Conversely, East Asia commonly has highly active forms of virulence factor genes *vacA* and *cagA* in the dominant *H. pylori* strain, which contributes to the fact that this area has the highest incidence of gastric cancer in the world [105]. Additionally in Asia, the *cag*PAI is present in almost all *H. pylori* infections [106], but is present in around 60-70% of strains in Europe [107]. These geographic differences in the predominant *H. pylori* strains in an area have been linked to patterns of human migration, with ancient lineages diverging into

at least 7 major ancestral populations that all modern strains can be traced to [104]. Sequencing analysis has shown that the bacterial genetic diversity is at its highest in East Africa and decreases with geographic distance, mirroring the spread of humanity from this region approximately 60,000 years ago [108]. From there it established itself with its hosts on every settled continent.

These genetic differences that can account for variations in disease prevalence are also important for considering treatment options, as they dictate antibiotic resistance rates as well. The role of genetics in *H. pylori* resistance rates will be discussed in greater detail in later chapters.

1.4.2.2 Host Factors: Age, Gender, Diet, and Socioeconomic Factors.

Adults are statistically more likely to show signs of *H. pylori* disease than children. Although the infection is often acquired in childhood, disease progression can be slow. Symptoms take a long time to become apparent, so disease is more commonly seen in people over the age of 30, with ulcer disease being most commonly seen in 30- to 40-year-olds, and gastric cancer seen more often in people aged over 50. There are also declining rates of *H. pylori* in

many countries, meaning that younger generations are less likely to have the infection at all. This could be due to better healthcare access in some parts of the world, and increased use of antibiotics, particularly in childhood where the infection is more easily cleared [109]. Additionally, because infection is usually lifelong if no treatment is provided, there is a cumulative effect that means that the infection rate is compounded with age. Unfortunately, treatment success rates are also declining due to more recent increases in antibiotic resistance in *H. pylori*, which means that although initial infection rates might be decreasing, disease is still a major concern.

Higher socioeconomic status and access to education are major predictors of *H. pylori* incidence within a population and should be considered when analysing other environmental factors [110]. Incidence of infection between males and females is not a settled topic, as some reviews have found infection rates to be highly similar across gender [109], while others have found a more frequent infection rate in males [111]. However, for disease, gastric adenocarcinoma is far more common in men. Location and smoking history do not account for this difference, and the mechanisms behind this phenomenon are as yet not fully understood [112].

1.5 *H. pylori* Virulence Factors

As previously mentioned, a major factor in the development of *H. pylori* related diseases is the complement of virulence factors displayed by the bacteria. The two virulence factors of major concern are the presence or absence of the *cag* pathogenicity island, and the genotype of the vacuolating toxin *vacA* that is present, as shown in **Figure 5**.

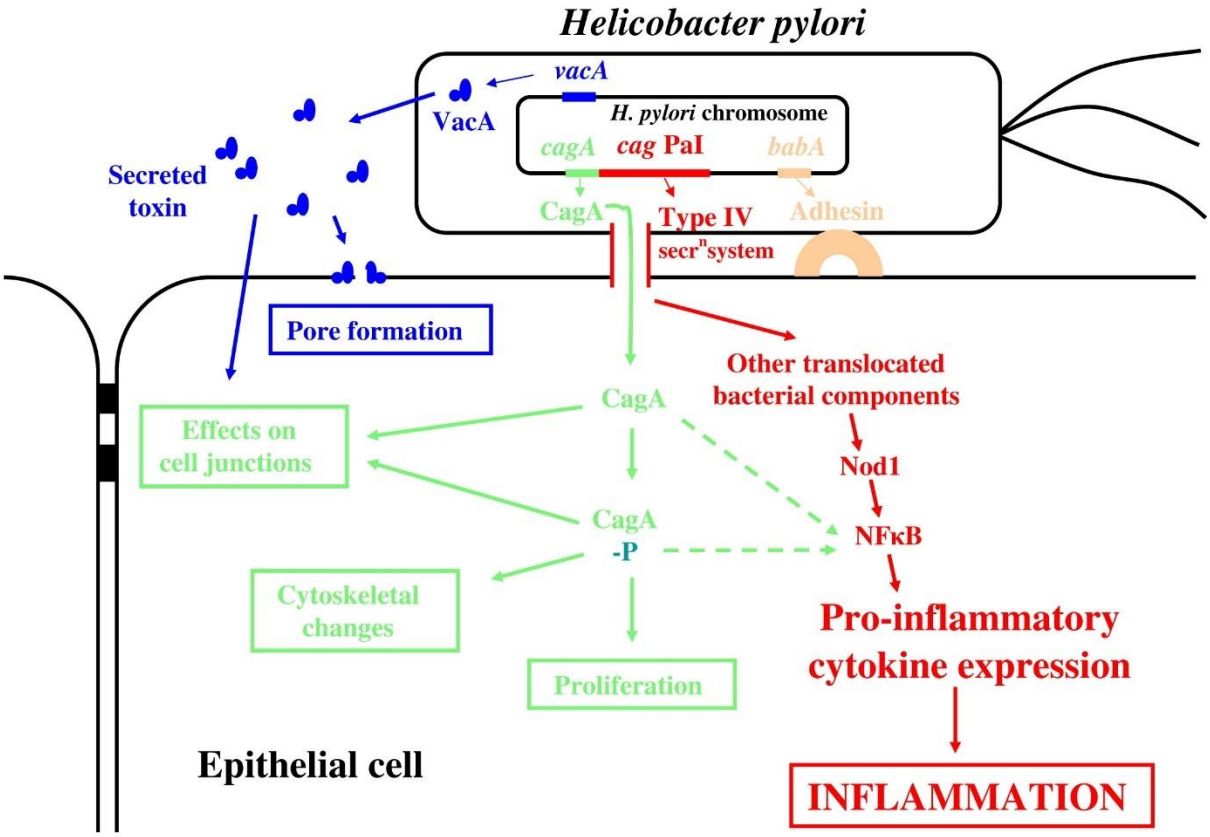


Figure 5 *- The effects of the major H. pylori virulence factors on host epithelial cells.*

The type IV secretion system shown in red is encoded by the cag pathogenicity island, which allows bacterial proteins such as CagA (green) and other factors to enter the host cell. These lead to the expression of pro-inflammatory cytokines, and cause inflammation and cytoskeletal rearrangement. The bacterium attaches to the epithelial cell via babA encoded adhesion factors, shown in orange. VacA, encoded by the vacA gene (blue) are released from the bacterial cell and can form pores within the membrane of the host cell, and can lead to the development of fluid filled vacuoles within the host cytoplasm. Image adapted from Robinson et al., 2007 [113].

1.5.1 VacA

The virulence gene *vacA* is always present in some form within the *H. pylori* genome, suggesting it may be an essential component of *H. pylori* dissemination or colonisation. The gene *vacA* encodes the protein VacA, a toxin that can enter human epithelial cells, and form membrane pores and cytoplasmic vacuoles. This also makes it useful for confirming *H. pylori* presence via PCR - but it can occur in different allelic variants, some of which are more highly associated with disease progression than others. There are three main sections of sequence diversity in the VacA protein (**Figure 6**), each with two major polymorphs: the signal (s1/s2), intermediate (i1/i2), and mid (m1/m2) regions [114].

Of these polymorphic forms, two are of particular interest when looking at disease outcome. The signal (s) region is related to signal peptides and is significantly linked to the secretion and vacuolating activity of the VacA toxin. The s1 form is an active form that can cause vacuole development, while s2 types have an

additional 12 hydrophilic amino acids, which blocks the VacA toxic activity and reduces occurrence of inflammation and ulceration [115]. Additionally, the s1 allele seems to be correlated to the presence of other virulence factor genes in the genome, such as the *cag*PAI and *babA* genes [116], [117], whereas the majority of s2 type strains are *cag*PAI negative [118].

In the intermediate region of the VacA toxin, the i1 form has been found to have a strong association with gastric adenocarcinoma [68] but there are no differences in inflammation or colonization between strains with i1 and i2 types [119]. The s1/i1 genotype combination is considered 'more active' and highly associated with gastric adenocarcinoma. However, in mouse models the less active s2/i2 form was more effective at colonising the stomach than the s1/i1 form [119], which may provide an explanation for the continued persistence of this allele combination. The mid region genotype is related to host cell receptor binding, as m1 and m2 have exhibited specificity towards different cell types [120], and m1 type *vacA* is able to initiate vacuolisation in a larger variety of epithelial cells *in vitro* [121]. As such, the s1/i1/m1 combination of *vacA* alleles can lead to more serious disease presentation and in a wider variety of host cells, making them important genetic predictors for gastric cancer and PUD. Allele occurrence varies in

different parts of the world, and this is reflected by the differing rate and severity of disease [122].

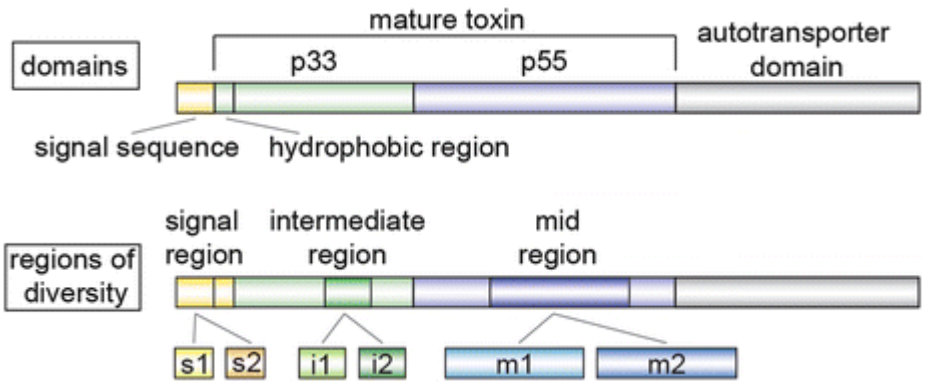


Figure 6 *- The domains and regions of diversity present within the VacA virulence factor in H. pylori.*

The domains are made up of a signal sequence region, as well as a section that is cleaved into two subunits (p33 and p55) that form the mature toxin, and an autotransporter domain that allows the toxin to move through the cell membrane. There are three main allelic variations possible within the VacA protein; the s (signal), I (intermediate), and m (mid) regions, which have two major variants each. The '1' variety of each allele can lead to more virulent activity and is linked to disease development. Image taken from Cover et al., 2016 [123].

The *vacA* gene encodes a 140 kilodalton (kDa) protein, which is cleaved into both the VacA toxin itself, and a β-barrel that is used as an autotransporter that can translocate the toxin across the outer membrane of the bacteria [124]. The section that forms the VacA toxin itself is an 88kDa protein consisting of two subunits (p33 and p55), that is produced and secreted by the bacteria once colonisation has occurred (**Figure 7**). The β-barrel domain sits across the bacterial outer membrane, and allows the VacA toxin to exit the cell, and bind to the outside of a host cell [125]. The host

cells then take in the toxin via endocytosis, where it causes the formation of large voids or vacuoles [126] which are the consequence of the VacA pores causing osmotic pressure to fill the host cell vesicles with cytoplasmic fluid [127].

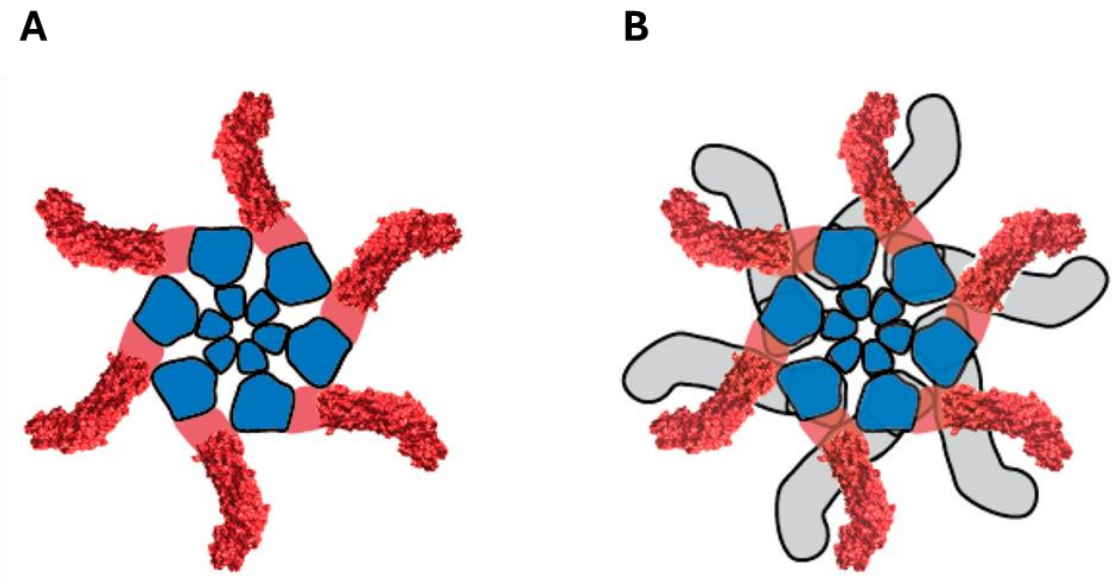


Figure 7 *– Pores formed by the assembly of p33 and p55 subunits of the VacA protein.*

The VacA p33 (blue) and p55 (red) subunits can assemble into oligomers such as the A) single-layered hexamer and B) double-layered dodecamer. The single-layered pore structure is more likely to incorporate into lipid membranes, and allow the movement of cellular fluid through, causing the formation of vacuoles within the host cell and disrupting cellular functions. Image taken from Foegeding et al., 2016 [124].

There is also evidence to suggest that VacA, once inside the cell, can cause a delay in cell apoptosis, allowing infected cells to persist. Even before VacA is taken up into the cell, it has been shown to be able to modulate cellular function, including increased production and translocation of mitochondrial proteins. The exact mechanisms of pathogenesis are unclear, but this interaction has

been associated with an increase in disease severity in mouse models [128]. The prevalence of the type of *vacA* present within a *H. pylori* strain varies by geography, and it is possible to have different *vacA* isotypes present within the antrum and corpus of the same individual [129]. The combination of s1m1 *vacA* isotypes are also significantly linked with the presence of the *cag*PAI, forming an extremely virulent disease-causing strain [130].

1.5.2 CagA

The virulence factor CagA is an onco-protein, encoded by the *cagA* gene, and is a major cause of the development of peptic ulcers and gastric adenocarcinoma in those infected with *H. pylori* [131]. The *cagA* gene is part of the *cag* pathogenicity island, which is a 40kb DNA insertion element [132]. The *cag*PAI also includes around 30 genes that encode the components of the type IV secretion system (T4SS) that allows CagA to be translocated into the host cell [1], [133], [134]. Type IV secretion systems are transporter units that act like a hollow needle, allowing proteins and other virulence factors produced by the bacteria to be moved into other cells such as host epithelial cells. Once inside, CagA can lead to a change in the morphology of host cells, caused by a rearrangement of the cytoskeleton that elongates the cell into a 'hummingbird' phenotype [135]. Additionally, it can disrupt the junctions between

cells and interfere with normal cell proliferation. Both CagA and the T4SS can also independently cause inflammatory responses in the host cells [136], [137].

1.5.3 CagA dependent and independent signalling

1.5.3.1 CagA Independent Signalling

The two means by which the *cag*PAI proteins interact with the host cells are known as CagA dependent and CagA independent signalling. CagA independent signalling is initiated when the T4SS comes into contact with a host cell. In *H. pylori,* the T4SS is made up of a series of Cag proteins encoded by the *cag*PAI that spans both bacterial membranes across to an adjacent host cell membrane. The end of the T4SS pilus consists of CagL proteins that interact with integrins protruding from the host cell surface. CagL binds to these proteins that span the host membrane, particularly integrins α5β1 and αVβ6, which when activated cause a series of responses within the cell [138]. There are two main signalling cascades that both lead to the release of the pro-inflammatory cytokine interleukin-8 (IL-8), which can happen even without CagA being translocated into the cell [137].

The Raf-MEK-ERK pathway consists of the serine/threonine kinase Raf, Mitogen-activated protein kinase (MAPK) kinase (MEK), and

extracellular-signal-regulated kinase (ERK) [139]. Each protein in the chain activates the next via phosphorylation. This leads to the upregulation of the transcription factor NF-κB which causes the induction of pro-inflammatory cytokines as shown in **Figure 8**. The Raf-MEK-ERK pathway can be triggered by a range of kinases such as the tyrosine kinase Src, Focal Adhesion Kinase (FAK) [140] and a GTP binding protein Ras, all of which in turn are activated by CagL binding to α5β1 integrin. Another CagA-independent route leads to the activation of transcription factor AP-1 via the c-Jun N-Terminal kinase (JNK) pathway, again leading to IL-8 activation and inflammation [141].

1.5.3.2 CagA Dependent Signalling

In the CagA-dependent route, the host integrins binding to CagL cause the translocation of CagA and peptidoglycan through the pilus and into the host cell, where they can interact with several different host signalling pathways and cause activation of pro-inflammatory cytokine IL-8 [140], [142]. Once CagA has entered the cell from the T4SS, it will either remain as it is, or become phosphorylated: both forms are able to interact with the host cell apparatus. Phosphorylated CagA activates the Raf-MEK-ERK pathway, which has been described previously. Phosphorylation occurs as a result of Src family tyrosine kinases (SFKs) interacting with CagA at the site of a highly conserved chain of 5 amino acids

(Gly-Pro-Ile-Tyr-Ala) called the EPIYA motif. The sequences surrounding the EPIYA motif can vary, and come in 4 different main varieties (A,B,C and D), which can influence the downstream signalling that occurs when CagA becomes phosphorylated, and will be discussed in more detail in the next section [135].

CagA can also function within the host cell without undergoing phosphorylation. Morphological changes stemming from CagA interacting with c-Met hepatocyte growth factor (HGF), independent of tyrosine phosphorylation, have been reported. Tight junction disruption has also been demonstrated, and both of which can play a role in carcinogenesis [143]. Translocated *H. pylori* peptidoglycan also interacts with NOD1 (nucleotide-binding oligomerization domain 1), a pattern recognition receptor within the cytoplasm. NOD1 associates with the protein RICK (RIP (receptor interacting protein)-like interacting CLARP (caspase-like apoptosis-regulatory protein) kinase) and leads to IL-8 induction via the transcription factors Activator Protein-1 (AP-1) and NF-κB [137].

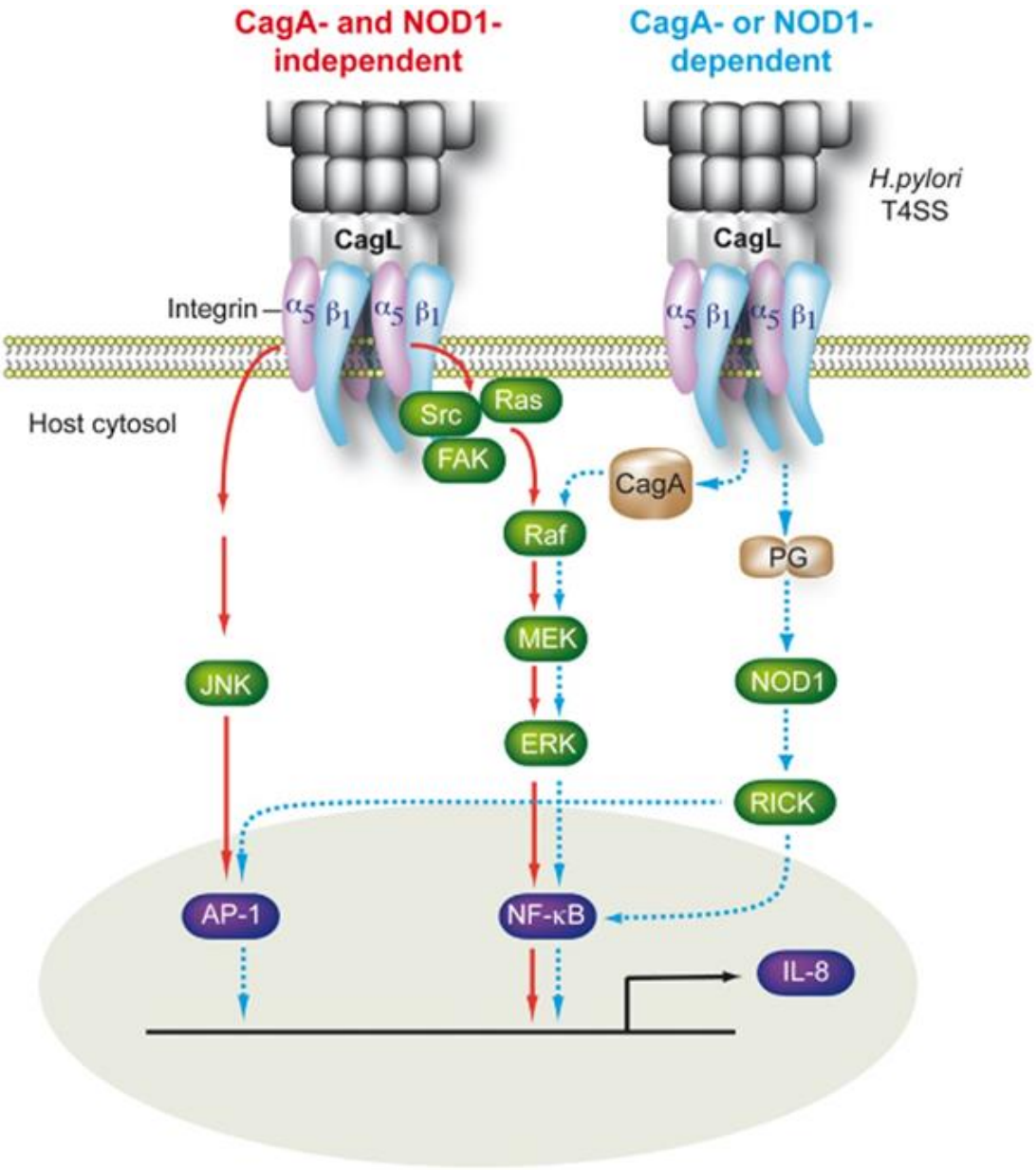


Figure 8 - *CagA and NOD1 dependent and independent pathways of inflammation via the type IV secretion system.*

CagL can bind to integrins in the host cell membrane, particularly α5β1. The binding of CagL to host cell integrins can cause upregulation of IL-8 directly, as well as indirectly, by translocating CagA and peptidoglycan into the host cell. The CagA/NOD1 dependent route (blue arrows) and the CagA independent route (red arrows) are shown here. CagA/NOD1 dependent route (Blue arrows); PG (Peptidoglycan) and CagA are translocated into the cell. PG interacts with pattern recognition receptor NOD1 (nucleotide-binding oligomerization domain 1), which interacts with RICK (RIP (receptor interacting protein)-like interacting CLARP (caspase-like apoptosis-regulatory protein) kinase) and leads to IL-8 induction via the transcription factors Activator Protein-1 (AP-1) and NF-κB.

CagA activates the Raf-MEK-ERK (Mitogen-activated protein kinase (MAPK) kinase (MEK), and extracellular-signal-regulated kinase (ERK)) pathway, activating NF-κB. CagA/NOD1 independent route (red arrows); CagL binding to α5β1 integrins activates Src, ras and FAK (Focal Adhesion Kinase) kinases, which initiate the Raf-MEK-ERK pathway. JNK (c-Jun N-Terminal kinase) phosphorylates AP-1 (Activator Protein-1), leading to IL-8 upregulation.
Image taken from Gorrell et al., 2013 [137].

1.5.4 EPIYA Motifs and Virulence

The phosphorylation of CagA within host cells can be linked to host disease and varies depending on *H. pylori* strain due to differences in the C-Terminal EPIYA motif of CagA, which is the amino acid sequence Glu-Pro-Ile-Tyr-Ala where phosphorylation occurs. Differences in the amino acid sequences surrounding the EPIYA motif determine what forms of the motif are present, and have been categorised as A, B, C and D (**Figure 9**). The combination of EPIYA motifs impact which signalling molecules are phosphorylated within the host cell, and in turn which signalling pathways are activated [135].

Many strains of *H. pylori* from Western countries carry a CagA protein with an EPIYA-ABC structure. The EPIYA-repeat region consists of one instance each of EPIYA-A and EPIYA-B, and then between 1 and 3 repeats of EPIYA-C. East Asian strains, however, are more likely to consist of EPIYA-A, -B and -D (**Figure 9**). The ABD form of the EPIYA motif more strongly activates host tyrosine

kinases such as Src homology region 2 domain-containing phosphatase (SHP-2) [144]. SHP-2 in turn activates MAPK signalling leading to the inactivation of focal adhesion kinases, which are a major component in host cell structure and motility pathways [145]. The disruption of these pathways is linked to cytoskeletal rearrangement of the cell causing an elongated hummingbird phenotype, inappropriate cell migration, and increases production of pro-inflammatory cytokine IL-8 [146]. CagA containing more repeats of EPIYA-C have also been shown to increase phosphorylation activity and has been linked to an increased risk of gastric cancer compared to Western-type CagA strains with only one EPIYA-C motif [147].

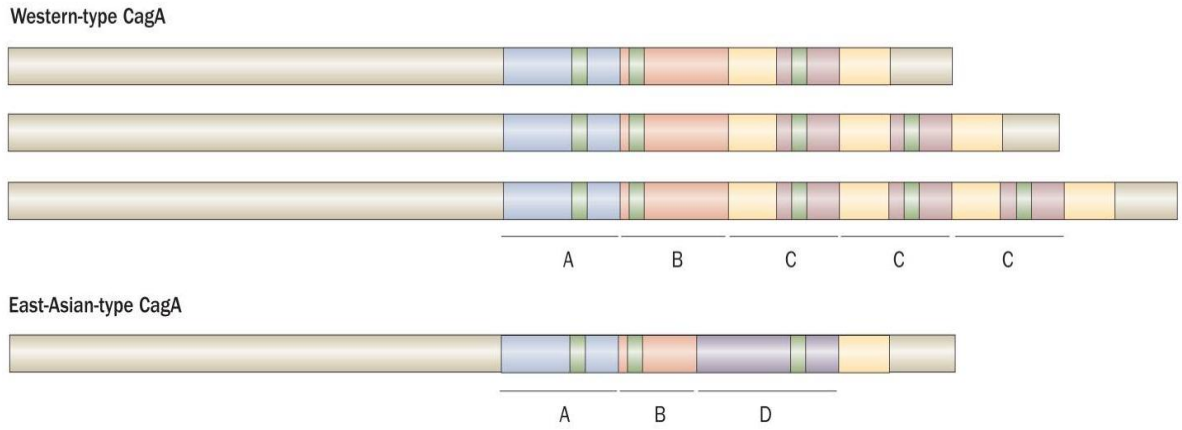


Figure 9 - *Schematic of the domains that make up the Western-type and East-Asian-type CagA protein.*
The green highlighted sections are the Glu-Pro-Ile-Tyr-Ala (EPIYA) sites of tyrosine phosphorylation, while the surrounding amino acids determine the EPIYA type (A, B, C and D). All CagA types contain EPIYA-A and -B, followed by either 1-3 C domains, or a D domain. In Western-type CagA, EPIYA-C occurs with between 1 and 3 repeats, whereas East-Asian-type CagA more commonly has an EPIYA-D motif instead.
Image taken from Yamaoka, 2010 [148].

When injected into the host cell, CagA can interact with mitochondria and cause it to activate the apoptosis pathway, modified to a sub-lethal level. It has also been found to downregulate mismatch repair of DNA, leading to an accumulation of mutations in the host nuclear DNA and mitochondrial DNA [149]. The CagA protein induces the release of host cytokines and chemokines by activating an alternative NF-kB pathway. Bacterial cell components such as muropeptides also activate host cell pattern recognition receptors. The mitochondrial apoptosis sequence is triggered, which begins to damage cell DNA and cause inflammation, however, the activation of apoptosis is often not absolute. This leads to cells that do not die as they normally would under apoptotic conditions, but are instead continuously exposed to damage and inflammation, which can lead to the development of gastric disease and cancer [150]. VacA and CagA also work in concert with one other, as VacA can lead to the accumulation of CagA proteins within host cells, furthering the effect [151].

1.6 Treatment

Treatment of *H. pylori* infection in the UK usually involves a regimen of at least 3 different antibiotics along with a proton pump inhibitor to reduce stomach acidity. European guidelines concerning

the diagnosis and treatment of *H. pylori* have been published by the Maastricht VI/Florence Consensus [79], as well as by the UKHSA [5]. Both of these sets of guidelines stress the importance of diagnostic testing and determining patient history, particularly any previous use of antibiotics. This is because earlier exposure to antibiotics that are then used in a treatment plan is a risk factor for harbouring resistant strains (particularly in the cases of metronidazole and clarithromycin) [152], and potential treatment failure. In cases of peptic ulcer disease, eradication of any concurrent *H. pylori* infection is the foremost recommended treatment. Immediate treatment is also recommended as a preventative measure against the development of gastric MALT lymphoma. Unfortunately, failure rate of *H. pylori* treatment regimens has increased in recent years, causing continued (and potentially progressed) disease presentation for patients, and the need for them to go through additional eradication therapy – sometimes multiple times [153].

Another reason for treatment failure is a lack of patient compliance. Treatment guidelines recommend a triple or quadruple therapy for patients for 14 days, with PPI being taken twice a day and antibiotics being taken once or twice per day, depending on the antibiotic. A typical regime can include the patient taking

between 6 and 12 pills per day, and this frequency and complexity has the potential to lead to incomplete or incorrect adherence to the treatment regime [154]. Lack of compliance is further exacerbated by potential side effects of the medication, which can cause more discomfort than the disease for which the patient is being treated. In particular, metronidazole can often cause symptoms of gastric irritation, sickness and diarrhoea, and a metallic taste in the mouth during the treatment course [155]. One study found that if only 60% of the medication in a treatment regimen is taken by the patient, eradication rates can drop by as much as 25% [156]. Combined with increasing rates of antimicrobial resistance in *H. pylori*, treatment success is becoming more difficult to achieve. The addition of bismuth to the treatment regime (known as quadruple therapy) has been shown in some cases to overcome the effect of antibiotic resistance on treatment success when regimes are closely followed by patients [157], however, continuously increasing the complexity of treatment regimens is not a long-term solution to the problem of antimicrobial resistance.

1.6.1 Antimicrobial Resistance

Globally, both primary and secondary resistance rates to antibiotics used in first- and second-line *H. pylori* treatments are increasing,

and with that comes increased likelihood of treatment failure. This demonstrates a need to reconsider treatment options based on the local failure rates and highlights the need to maintain good standards of antibiotic resistance testing in *H. pylori* around the world in order to elucidate these trends. Resistance in *H. pylori* to many widely used antibiotics has in general been on the rise, but there has been a significant increase in incidences of resistance to clarithromycin, metronidazole, and levofloxacin over the last decade, while tetracycline and amoxicillin resistance rates have remained relatively unchanged [158]. This will be delved into more deeply in the subsequent chapters of this thesis.

Correct and informed treatment of *H. pylori* is essential for patient well-being and improved prognosis, and this can only be achieved with accurate and up-to-date information on the local antibiotic resistance rates for *H. pylori*. Therefore, gathering that data has been a key aim of this research project, as well as investigating techniques that can determine antibiotic resistance profiles in alternative and more efficient ways, by examining the genetics of *H. pylori* antibiotic resistance. Exploring genetic sequencing procedures has the potential to reduce patient wait time for effective treatment and increase the accessibility and ease of obtaining relevant disease information for clinicians.

1.7 Thesis Aims

Antimicrobial resistance in *H. pylori* is a growing problem worldwide and can lead to treatment failure. Correct identification of resistance and knowledge of local antibiotic resistance rates can improve eradication success and reduce the risk of the development of secondary antibiotic resistance. However, antibiotic susceptibility testing in *H. pylori* isolates is a slow process and is rarely carried out on clinical isolates.

Therefore, the aims of this study are focused on collecting local antibiotic resistance rates to *H. pylori and* identifying trends in resistance that can be used to help inform treatment protocols. Additionally, another main aim is to look at the ways in which antibiotic resistance is identified. Currently, it is difficult to predict phenotypic resistance in *H. pylori*, as genotypic data is not always indicative of whether or not a certain antibiotic will be effective in treatment. Different methods of sequencing can be used to try and identify antibiotic resistance genes, however, there are benefits and drawbacks to both Illumina and MinION whole genome sequencing when trying to identify single nucleotide polymorphisms (SNPs) that may confer resistance. Gathering sequencing data via a variety of methods and comparing the results to the antibiotic susceptibility data of the Nottingham Strain Collection will allow for

greater understanding of how sequencing could be used as a tool to inform patient treatment options.

The key targets of this study are to assess the antibiotic resistance profiles of clinical *H. pylori* isolates from the Nottingham Strain Collection to 5 antibiotics (amoxicillin, tetracycline, clarithromycin, levofloxacin, and metronidazole). Using these data, and additional patient and genotypic data previously obtained, there are several hypotheses that can be tested concerning the relationship between antibiotic resistance and a variety of factors. As such, the hypotheses for this study are as follows:

1. **Assess the antibiotic resistance profiles of the Nottingham Strain Collection.**

Routine testing of *H. pylori* clinical isolates for antibiotic resistance is not commonly carried out. Guidelines suggest consulting local resistance rates to inform treatment plans however this information is unavailable in many areas. Therefore, the aim for this section is to characterise the antibiotic resistance profiles of the *H. pylori* strains from the Nottingham Strain Collection to 5 antibiotics commonly used in its treatment (amoxicillin, tetracycline, clarithromycin, levofloxacin, and metronidazole). The data could then be used to identify trends or relationships in local antibiotic resistance rates to various factors, such as virulence

factors of the strains, patient demographics, and year in which the isolate was collected.

2. **Identify the mechanisms and relationship between phenotypic and genotypic antibiotic resistance to levofloxacin in *H. pylori*.**

The relationship between genotypic and phenotypic levofloxacin resistance is not fully understood. Certain single nucleotide polymorphisms in the *gyrA* gene have been linked to increased levels of phenotypic resistance to levofloxacin, but the specifics of how these polymorphisms relate to resistance is unclear. This means that sequencing of *H. pylori* is not enough to accurately predict levofloxacin resistance and as such can't be used for informing disease treatment. Therefore, another research aim for this study involves sequencing the *gyrA* gene of *H. pylori* isolates with a range of levofloxacin resistances, based on the minimum inhibitory concentration (MIC) data already gathered. Any potential resistance-conferring polymorphisms are then identified and compared to the MIC data, with the aim to elucidate the relationship between levofloxacin genotypic and phenotypic resistance, and then to look further at the possible mechanisms behind this.

3. **Assess the suitability of different sequencing techniques for identifying SNPs related to antibiotic resistance in *H. pylori*.**

Identification of resistance in *H. pylori* is a slow process that is rarely carried out in clinical settings. A potential solution to this would be to sequence isolates and identify resistance genes, however many whole genome sequencing techniques do not reliably identify the presence of SNPs that are associated with antibiotic resistance. Therefore, another aim of this study was to perform whole genome sequencing of *H. pylori* isolates using two different techniques: Illumina and MinION sequencing. These two methods of sequencing both have strengths and weaknesses in terms of identifying SNPs, and so a further aim was set to see if a technique could be developed to combine the two sets of sequencing data and improve the reliability of SNP detection. An additional aim was to compare this hybrid sequence data to the Sanger sequencing *gyrA* results obtained previously, and identify how useful each of these techniques may be for determining eradication therapy options for clinicians that are best suited to the individual patients.

Chapter 2: General Materials and Methods

2.1 Characterisation of the Nottingham Strain Collection

2.1.1 Ethics and Biopsy Sample Collection, Isolation, & Storage

The Nottingham Strain Collection comprises 284 *H. pylori* isolates gathered from 162 patients attending Queen's Medical Centre (Nottingham, UK) for a routine upper gastrointestinal endoscopy, between 2001 and 2018. The patients all gave written, informed consent for collection of additional gastric biopsies for research purposes, as well as peripheral blood samples. Anonymised demographic information was gathered, which included age, sex, and smoking habits. *H. pylori* infection, and gastro-duodenal disease status was also collected (**Table 1**). The Nottingham Research Ethics Committee 2 granted ethical approval for the use of the clinical samples and patient information for this study (reference 08/H0408/195). Patients regularly taking high-dose NSAIDs or antibiotics in the preceding two weeks were excluded from the study.

H. pylori infection status was determined using the rapid biopsy urease test, and confirmed by histology, successful isolation of the strain from biopsy tissue, serology, and PCR. All patients had been referred for an endoscopy by their general practitioner (GP), and the most common indication for referral was chronic dyspepsia. Biopsy samples were taken from both the antrum and corpus regions of each patient's stomach. Culturing of the initial *H. pylori* isolates from the pinch biopsies was performed by Professor John Atherton and his colleagues, as previously described [159].

To isolate *H. pylori* samples, biopsies were plated onto 5% (v/vol) horse blood agar base no. 2 plates (Oxoid, UK). These were incubated for 48-72 hours at 37°C, in microaerophilic conditions (10% carbon dioxide, 5% oxygen, 85% nitrogen). Avoiding contaminant growth, colonies and sweeps of *H. pylori* were identified and picked based on morphology and confirmed using Gram staining and urease testing. Colonies from the same patient biopsy sample were pooled and were then stored at -80°C in a suspension of Iso-sensitest Broth (Oxoid, UK) with 15% (v/v) glycerol (Courtin & Warner, UK). Patient notes gathered by the endoscopist included information of whether the patient had previously undergone *H. pylori* eradication therapy, and how many rounds of treatment they had received. However, information concerning the patient history of specific antibiotic prescriptions was held by patient’s GPs, and unavailable for our access.

2.1.2 Patient Demographics

Table 1 *- Demographics of the 162 patients from who the H. pylori isolates were derived.*

Gender	Male 50.0% (n=81) Female 50.0% (n=81)
Age	Mean 53.2 years (range 19-86 years)
Gastro-duodenal disease status identified at endoscopy	Duodenal ulcer 38.9% (n=63) Gastric ulcer 11.1% (n=18) Gastric cancer 0.6% (n=1) Gastritis/duodenitis 6.8% (n=8/3) None 42.6% (n=69)
Previous failed *H. pylori* treatment	25.3% (n=41)
Isolates recovered from antral and corpus gastric biopsies	46 isolates from the antrum only (single site isolates from 46 patients) 37 isolates from the corpus only (single site isolates from 37 patients) 158 isolates, from both the antrum and corpus (paired isolates from 79 patients) Total of 241 isolates recovered from 162 patients
Smoking, % (n/N)	Non-Smoker 72% (116/162) Smoker 15% (25/162) Ex-smoker 9% (14/162) Unknown 4% (7/162)
Virulence factor genotype of all isolates, % (n/N)	*cagA* status: Positive 72% (166/232); Negative 28% (66/232) (9 isolates removed due to ambiguous data) *vacA* type: i1 66% (152/231); i2 34% (79/231) (10 isolates removed due to ambiguous data)

2.2 Histological determination

Histological status of the samples was determined by analysis of additional biopsies that were taken from the same region of the

patient's stomach, at the same time as biopsies taken for *H. pylori* isolation. To assess histology scores, biopsy samples were fixed in formalin immediately after collection and embedded in paraffin wax. The 4μm sections were then prepared with a microtome, mounted on glass slides, and stained with either toluidine blue, or haematoxylin and eosin. Assessment of histopathology was performed by Dr Richard Ingram and Dr Abed Zaitoun (consultant gastrointestinal histopathologist), measured using the Sydney scoring system [160].

2.3 PCR Genotyping of *vacA* Isotype and *cagA* Status

Isolates were tested for the virulence factors *vacA* i1 and i2 isotype, as well as *cagA* positive or negative status via PCR. This was performed by Joanne Rhead and other members of the research group, using methods as previously described [68], [161].

2.4 Preparation of Frozen Stocks and Culturing

To prepare frozen isolates for testing, the cryo-tubes containing the stocks were thawed slightly, and 100μl of frozen stock was pipetted onto pre-warmed 5% (v/v) horse blood agar base no.2 plates (Oxoid, UK). The pipetted stock was spread over the agar until the surface had dried and was then incubated at 37°C for 48-58 hours

in microaerophilic conditions (5% carbon dioxide, 6% oxygen, 3% hydrogen, and 86% nitrogen). Bacterial growth was then picked from the outermost edges of the lawn with a sterile cotton swab and passaged onto two fresh no.2 horse blood agar plates and incubated for a further 24 hours in the conditions as described above. If a sample had low levels of bacterial growth after the first incubation, then available growth was passaged onto four no. 2 horse blood agar plates during the second incubation step, to maximise bacterial yield for later use.

2.5 Media Preparation

For susceptibility testing, batches of Mueller-Hinton 7.5% horse blood agar plates were created. 15.6g of Mueller-Hinton agar (Oxoid) was dissolved in 370ml deionised water and autoclaved. Once set, bottles of agar were taken as needed and melted in a 70°C water bath until no solid agar remained, and then cooled to 50°C in another water bath. This cooling step was done to minimise denaturation of blood proteins when adding horse blood. Defibrinated horse blood (Thermo Scientific) was warmed to 37°C, and then 30ml was added to the bottle of agar and mixed. 30ml aliquots of the blood agar were poured into sterile petri dishes (Oxoid, UK) and allowed to cool completely. Plates were stored at 4°C and used within 7 days. From each batch of plates made, one was tested for quality control. A suspension of *Escherichia coli*

strain DH5-α was prepared in sterile 0.85% saline (suspended to a standard of 3 ± 0.2 on a McFarland Den1 Densitometer), and a sterile cotton swab was used to spread the suspension over the surface of a plate and allowed to dry. A 10μg amoxicillin disc was placed in the centre of the plate, which was then inverted and incubated for 16 hours at 37°C, under normal atmospheric conditions. The diameter of the zone of clearing around the disc was then measured in millimetres using callipers. Batches of plates were only used if the zone diameter was 12-20 mm.

2.6 Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed using both E-test strips (bioMérieux, France) where possible, and a verified disc diffusion method. The disc diffusion method was performed based on a modified methodology as described in Lang *et al*, 2004 [162], and outlined in the section below. For both disc diffusion and E-test strip methods, *H. pylori* isolates were incubated for 24 hours, and then the growth for each strain was suspended in sterile saline (as described above) and spread onto 6 prepared blood plates using a swab.

2.6.1 Disc Diffusion Method

For the disc diffusion method, once the plates were dry, one antibiotic disc (either clarithromycin (15μg), amoxicillin (10μg), rifampicin (5μg), levofloxacin (5μg), tetracycline (30μg), or

metronidazole (5µg) (Oxoid, UK)) was placed firmly onto the centre of the plate using sterile forceps. For each set of sensitivity tests performed, a control strain of *H. pylori* NCTC 11637 (ATCC 43504/CCUG 17874) was also plated, and antibiotic discs were applied as described above. Plates were incubated at 37°C under microaerophilic conditions as stated above for five days, after which the diameter of the zone of clearing was measured in millimetres using callipers.

The zone of clearing was defined as the area around the disc with no bacterial growth. In cases where a second ring of inhibited growth was seen, both measurements were taken. Measurements for each individual plate were taken twice and an average calculated. All susceptibility tests were duplicated independently by a colleague. Plates that were overgrown with contamination were discounted and the test repeated. The breakpoints for antibiotic discs were taken from previously published literature (**Table 2**). Strains that had a zone of clearing diameter for metronidazole that measured between 16mm and 20mm were classified as having 'intermediate' resistance. These strains were retested with E-test strips to determine a clearer classification of resistance or susceptibility.

Table 2 *- Breakpoints used to determine strain resistance or susceptibility based on the diameter of the zone of clearing in mm. References indicate the source of the breakpoints chosen for this study.*

Antibiotic	Diameter of the zone of clearing, with breakpoint limits indicative of resistance and susceptibility		Reference
	Resistant	Susceptible	
Clarithromycin	≤28mm	>28mm	Alarcòn-Millán et al., 2016 [163]
Amoxicillin	≤25mm	>25mm	Lang et al., 2004 [162]
Rifampicin	<21mm	≥21mm	Glocker et al, 2007 [164]
Levofloxacin	<12mm	≥12mm	Yu et al, 2011 [165]
Tetracycline	<25mm	>25mm	Lang et al, 2004 [162]
Metronidazole	<16mm*	≥21mm*	Chaves et al, 1999 [166]

**Strains that had a zone of clearing diameter between these two measurements were classed as being of 'intermediate' resistance.*

2.6.2 E-test Strip Method

Once the plates were dry, sterile forceps were used to place one E-test strip in the centre of the agar - either amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin or

rifampicin. Gentle pressure was applied to the length of the E-test strip to remove air bubbles from under the strip, ensuring good contact with the surface of the agar. All antibiotic E-test strips had a concentration gradient of 0.016-256 µg/ml, except levofloxacin and rifampicin which each had a gradient of 0.002-32 µg/ml. Incubation conditions, repeats, and control strains were all the same conditions as used for the disc diffusion method. After incubation, the minimum inhibitory concentration (MIC) of each strain was determined by identifying the numerical marking printed on the E-test strip that the zone of clearing reached. Breakpoints indicating the resistance and susceptibility of the E-test strips were taken from guidelines published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 12.0 [167]. Each test was repeated independently by a colleague, and any strains that had conflicting or unclear results were tested again.

Table 3 *- Breakpoints used to determine strain resistance or susceptibility based on the point on the E-test strip reached by the edge of the zone of clearing.*

Antibiotic	**EUCAST E-test strip breakpoints (µg/ml)**	
	Resistant	**Susceptible**
Clarithromycin	>0.5	≤0.25
Amoxicillin	>0.125	≤0.125
Rifampicin	>1	≤1
Levofloxacin	>1	≤1

Tetracycline	>1	≤1
Metronidazole	>8	≤8

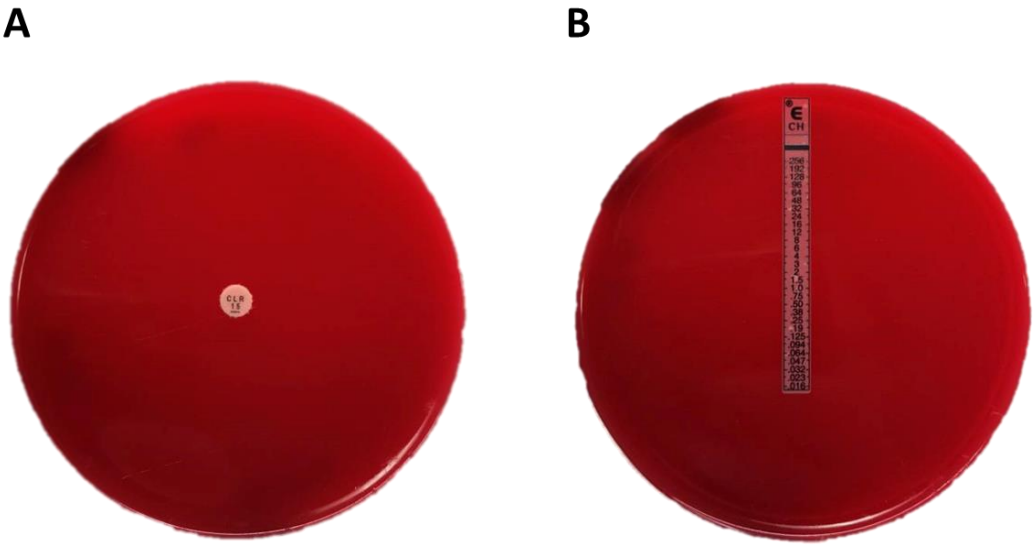


Figure 10 *- Mueller-Hinton agar plates supplemented with 7.5% defibrinated horse blood, swabbed with a H. pylori saline suspension and allowed to dry.*

A) Typical placement of a clarithromycin antibiotic disc in the centre of the plate.
B) Typical placement of a clarithromycin E-test strip.

2.7 Data Analysis and Statistical Tests

Resistance data was recorded using Microsoft Excel, and statistical analysis was performed using the software GraphPad Prism 9.5.1. A Chi-square test for trend was used for analysis of antibiotic resistance by year, and a Fisher's exact test was used for analysing the frequencies of resistance between those patients who had and

had not previously received *H. pylori* eradication therapy. P-values of $\leq$0.05 were considered statistically significant.

Chapter 3: Antibiotic Resistance in *Helicobacter pylori* Collected in Nottingham Between 2001 and 2018

3.1 Introduction

Effective *H. pylori* eradication therapy is needed to heal and prevent recurrence of peptic ulcers, to treat low-grade gastric B cell MALT lymphoma, and treat *H. pylori*-associated chronic dyspepsia, and also as an important part of a strategy to prevent gastric adenocarcinoma in high-risk groups [79]. Treatment is vital to the health and recovery of the affected patients, as once *H. pylori* has colonised the stomach, the infection rarely abates without intervention. Despite decades of research, there is no effective vaccine against *H. pylori* infection [168], meaning that currently antibiotics remain the only viable means of treating *H. pylori* infections [169].

However, antibiotic therapy is becoming less effective in some areas, due to an increase in antibiotic resistance rates. Because of this, some patients are exposed to multiple courses of antibiotics, which fail to treat the infection. In an effort to prevent this, treatment guidelines say local rates should be checked when deciding what course of treatment to use. Resistance rates to

different antibiotics can vary greatly between areas, so up-to-date regional data is a key asset for clinicians to have available.

The goal of the work described in this chapter is to examine antibiotic resistance rates in isolates taken from patients at Queen's Medical Centre, Nottingham, over a 17-year period. Additional information about both host and bacterial factors has also been analysed to identify how resistance might develop or persist in a population.

3.1.1 Current Treatment Recommendations and Clinical Guidelines

European Guidelines concerning the diagnosis and treatment of *H. pylori* have been published by the Maastricht VI/Florence Consensus [79] as well as by the UK Health Security Agency [170]. Both sets of guidelines emphasise checking patient history and encouraging compliance, in order to safeguard the continued effectiveness of the antibiotics, as each course of metronidazole and clarithromycin used by a patient increases the risk of the development of resistant bacteria [152]. The UKHSA guidelines recommend a 'Test and Treat' policy, meaning that eradication therapy should be the priority treatment after obtaining a positive *H. pylori* test result. For both the UKHSA and The Maastricht Consortium, treatment guidelines include options for first, second- and third-line therapies, which should be worked through

sequentially as necessary. These treatment regimens include combinations of antibiotics, PPIs to suppress acid production (specifically, lansoprazole, omeprazole, pantoprazole, or esomeprazole), and bismuth compounds. The selected antibiotic regimen depends on previous patient treatments, patient co-morbidities, local rates of antibiotic resistance, and the availability of treatments. It is recommended that if a patient has recently been treated with an antibiotic, that drug should not be used in their treatment for *H. pylori*.

As shown in **Figure 11** UKHSA guidelines describe first line treatment as a triple therapy of PPI twice daily, plus two antibiotics (amoxicillin and either clarithromycin or metronidazole) for 7 days. If this fails to eradicate the infection, a second line treatment is necessary. This includes a PPI with amoxicillin, in addition to a second antibiotic that was not included in the first round of therapy (i.e. clarithromycin or metronidazole). If the patient has previously been exposed to metronidazole or clarithromycin, the second line treatment includes either tetracycline hydrochloride or levofloxacin, with a PPI and amoxicillin. For patients who have a penicillin allergy, metronidazole is recommended instead of amoxicillin. A third line therapy combination usually includes 10 days of a quadruple therapy of a PPI, bismuth subsalicylate, and two of the above antibiotics that have not been used previously, or furazolidone or rifabutin.

In many places in the world, antibiotic resistance profiles of circulating *H*. *pylori* strains are sampled, and this has led most regional and national guidelines to recommend a move away from clarithromycin-based triple therapy to other regimens [79], [171], [172], [173], [174]. Unfortunately, this information is not available in the UK because there is no routine culture or screening of isolates, despite guidelines stating that this should be performed after two failed rounds of treatment. Without up-to-date *H. pylori* resistance data, patients can be exposed to multiple rounds of ineffective combination antibiotic therapy.

One large previous UK cohort study of *H. pylori*-infected adult patients, between February 2000 and May 2001, revealed that the overall success in eradicating *H. pylori* infection after one, two and three rounds of therapy was 73%, 94% and 98%, respectively [175]. Several different first-line therapy regimens had been used, the most common being combinations of a PPI with amoxicillin and metronidazole (which achieved 65% successful eradication), PPI with amoxicillin and clarithromycin (72%), or PPI with metronidazole and clarithromycin (66%). Recent data on *H. pylori* antimicrobial sensitivity in the UK, however, are lacking.

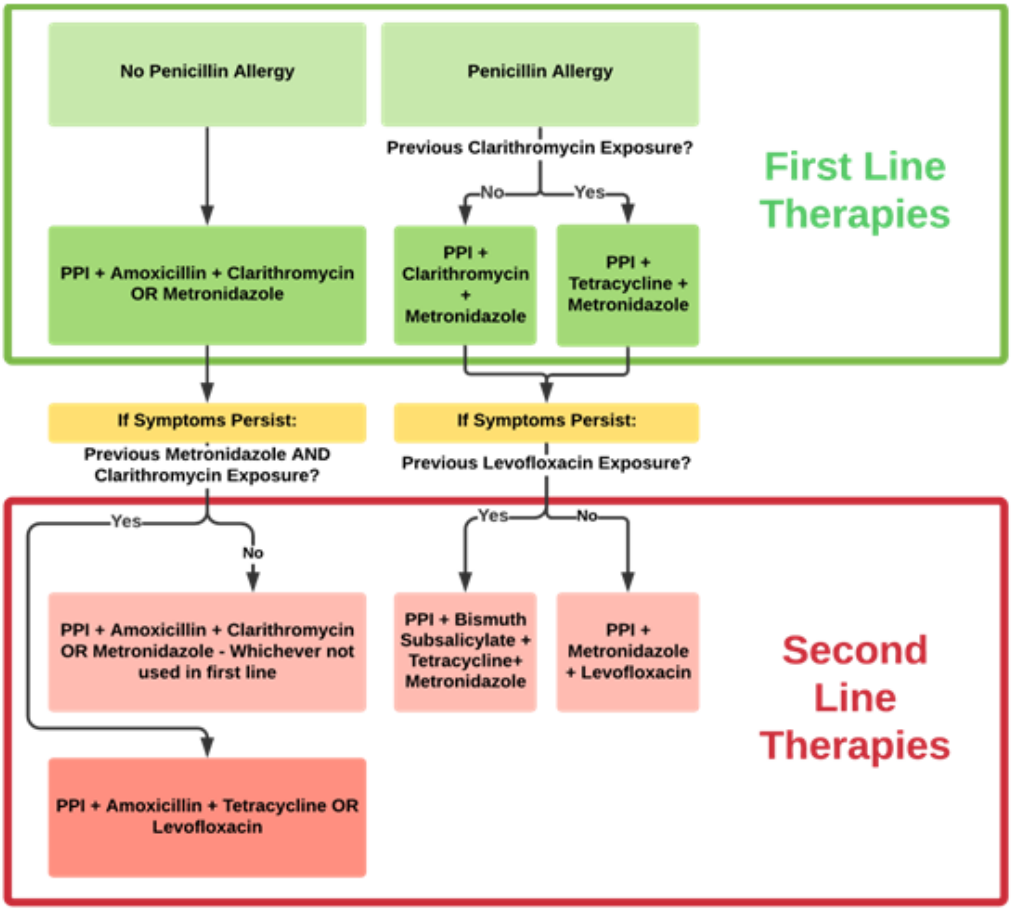


Figure 11 *- UK Health Security Agency guidelines for first- and second-line therapies for the treatment of H. pylori. PPI = proton pump inhibitor*

Bismuth and other heavy metals have been used to alleviate symptoms of dyspepsia for hundreds of years [176], and bismuth salts are now used in quadruple therapies to treat *H. pylori.* Compounds such as bismuth subsalicylate (i.e. Pepto-Bismol©) have been shown to work synergistically with antibiotics to reduce the bacterial burden and increase the likelihood of *H. pylori* eradication [177]. There have been no reports of *H. pylori* displaying resistance to bismuth treatments. Additionally, incorporating bismuth-based compounds into antibiotic treatment

regimens has been shown to reduce the development of antimicrobial resistance [178] and contribute to the eradication of antibiotic-resistant strains [179]. Although the mechanism of action of bismuth salts on *H. pylori* is not fully understood, there is evidence to suggest that bismuth disrupts several essential processes within the bacteria such as inhibition of metabolic enzymes, ATP production, and inducing oxidative stress. It also binds histidine rich proteins and heat shock proteins, contributing to reduced bacterial fitness and increasing the success of eradication therapy. Bismuth also acts as a protective layer over stomach ulcers and promotes healing by encouraging the secretion of prostaglandins and epidermal growth factors [177].

3.1.2 Antimicrobial resistance in *H. pylori*

Rates of primary and secondary *H. pylori* resistance to different antibiotics vary worldwide, depending on local strain prevalence and the various usage of antibiotics within each area. Primary resistance is defined as antimicrobial resistance in *H. pylori* strains that originated from patients that have not previously had exposure to antibiotic therapy, whereas secondary resistance is antimicrobial resistance in strains where the patient has undergone at least one failed round of antibiotic therapy [24]. A study in Japan found that an increase in the use of certain antibiotics over time corresponds with an increase in rates of resistance to those

same antibiotics, evidencing the argument that the use of an antibiotic drives the development of resistance to it [180]. Of particular concern are resistances shown to the antibiotics used in first- and second-line treatments, such as metronidazole, clarithromycin, amoxicillin, tetracycline (first-line therapies), and levofloxacin (a second-line therapy) [181]. Clarithromycin-resistant *H. pylori* in particular poses a threat to public health, and new treatment strategies are urgently needed [182].

3.1.3 Global Resistance Rates

Globally, both primary and secondary resistance rates to antibiotics used in first- and second-line *H. pylori* treatments are increasing, and with that comes increased likelihood of treatment failure. This demonstrates a need to reconsider treatment options based on the local failure rates and highlights the need to maintain high standards of antibiotic resistance testing in *H. pylori* around the world in order to elucidate these trends. Resistance in *H. pylori* to many widely used antibiotics has in general been on the rise, but the increase in resistance has been particularly significant for clarithromycin, metronidazole, and levofloxacin over the last 15 years, while tetracycline and amoxicillin resistance rates have remained relatively unchanged [158], [183].

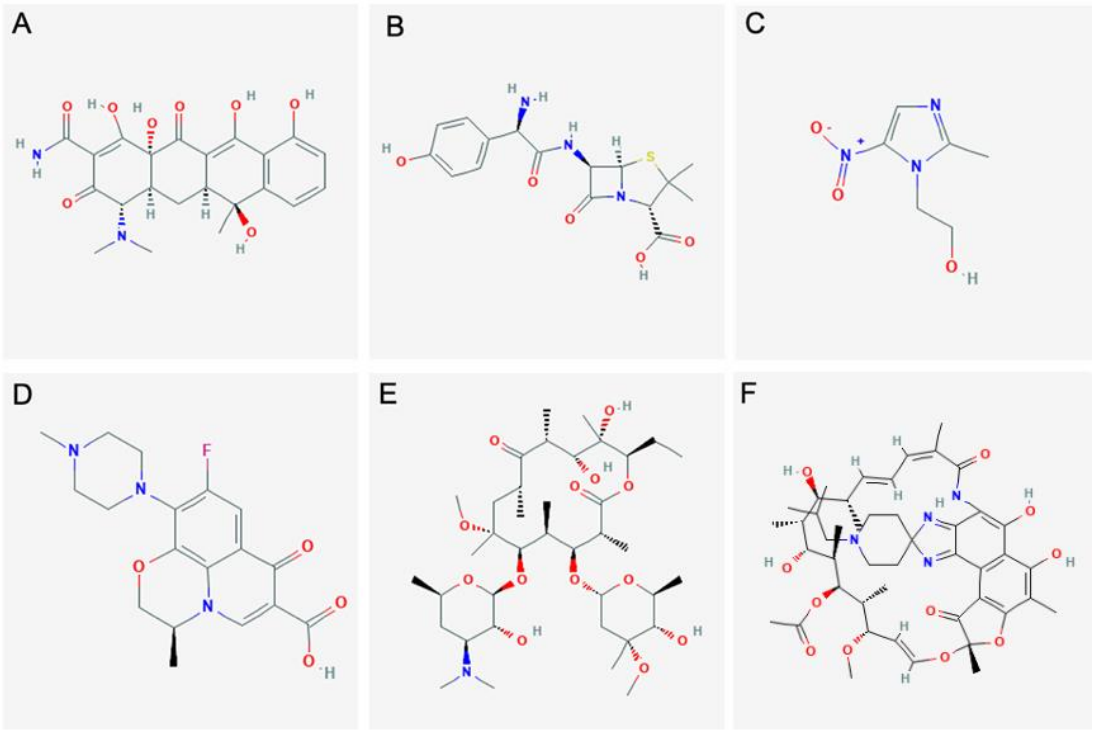


Figure 12 *- Chemical structures of six of the first- second- and third-line line antibiotics against H. pylori.*
Obtained from PubChem National Library of Medicine. A) Tetracycline B) Amoxicillin C) Metronidazole D) Levofloxacin E) Clarithromycin F) Rifabutin

3.1.4 Tetracycline

Overall, resistance to tetracycline is generally low (<10%) in most WHO geographic areas [24], however, in regions of Africa, resistance levels are as high as 48% [184]. Tetracycline is a broad-spectrum antibiotic that works by inhibiting protein synthesis in bacteria. It does this by blocking tRNA from interacting with the bacterial ribosome, halting growth and repair. The chemical structure consists of 4 linear fused rings with functional groups attached **Figure 12 A**. The 4 carbocyclic rings are the backbone

for each of the antibiotics in the tetracycline family and are required for antibiotic activity [185]. Resistance to tetracycline is mediated by mutations in the 16S rRNA gene, which encodes part of the bacterial ribosome. An amino acid substitution in the ribosome reduces tetracycline binding affinity and diminishes the efficacy of the antibiotic [186].

3.1.5 Amoxicillin

Although the European resistance levels to amoxicillin have remained low at ~0.5%, the rates in Africa (72.6%) [184] and South Asia (23%) [187] are much higher, although this can vary significantly between regions even within the same country [188]. Amoxicillin is a penicillin derivative, with an additional amino group attached. Structurally, amoxicillin contains the characteristic beta-lactam ring **Figure 12 B**, and functions by binding to bacterial penicillin-binding proteins. This interaction causes inhibition of transpeptidation, a process which is an essential part of bacterial growth. As bacteria grow, they break down sections of the rigid cell wall to insert extra peptidoglycan polymers. These polymers are secured together enzymatically by penicillin-binding proteins, completing the transpeptidation process. Normally, this allows for the expansion of the cell wall. However, if amoxicillin binds to the penicillin-binding proteins and blocks the formation of new cross-

link bonds between the molecules, the bacteria will instead lyse [189].

In other types of bacteria, the production of the β-lactamase enzyme is a common mechanism of resistance to amoxicillin. The enzyme functions by degrading the β-lactam ring that is present in all penicillins. Although there are examples of *H. pylori* isolates containing the β-lactamase gene [190], it is uncommon [191]. More frequently, amoxicillin resistance in *H. pylori* is conferred by mutations in the genes encoding the penicillin-binding protein, particularly PBP1A [192], PBP2 and PBP3 [193], as the changes in amino acid makeup reduce how well amoxicillin can bind to the protein [191].

3.1.6 Metronidazole

According to a 2018 review by the WHO, both primary and secondary metronidazole resistance levels far exceed the 15% threshold of resistance in every global region, the level at which alternative therapies are recommended. Metronidazole has by far the highest resistance rates for the first-line *H. pylori* antibiotics globally, with even the lowest regional prevalence at 38% in Europe, and the highest at around 91% in Africa [24]. Multi-drug resistance is also common, particularly the combination of metronidazole and clarithromycin [194]. This resistance

combination has a highly increased first-line therapy failure rate, with only 75% eradication success when using concomitant therapy compared to 90% eradication success when the strain is metronidazole and clarithromycin susceptible [195]. Metronidazole is used for the treatment of anaerobic bacteria, where it causes the degradation of the DNA helical structure, leading to strand breakage and cell death. Metronidazole is for the most part only effective within anaerobes, which produce the enzymes capable of reducing the antibiotic to its active hydroxylamine form once inside the cell. It is in the family of nitroimidazole antibiotics, so called because of the nitro functional group (**Figure 12 C**) [196].

Metronidazole resistance is conferred by mutations in the *rdxA* gene, with particularly high resistance found in strains that have a truncated version of the gene. The *rdxA* gene encodes an NADPH nitroreductase which catalyses metronidazole. Without a functional version of this protein, metronidazole remains in the inactive form and has reduced antibiotic activity [197].

3.1.7 Levofloxacin

Levofloxacin resistance rates have increased globally, with countries such as China, the USA and Italy all crossing the threshold of 20% resistance over the last two decades. In China particularly, resistance rates have been recorded as high as 34%

[198]. In a study spanning 2012 to 2016, the lowest measured regional resistance rate was 12% with most regions having rates of >20%. The Western Pacific Region especially has seen a significant increase from 12% to 31% between 2006 and 2016. However, the rate of resistance in the Eastern Mediterranean region has shown a reduction in that time, from 32% to 24% [24]. It should be noted that some studies use ciprofloxacin as opposed to levofloxacin to measure resistance rates, both of which are in the fluoroquinolone family, as there is considerable cross-resistance between the two [199] As a fluoroquinolone (so called because of the fluorine group at the R6 position (**Figure 12 D**)), levofloxacin functions by targeting DNA gyrase enzymes in bacteria which regulate DNA supercoiling during cell replication [200]. In *H. pylori*, fluoroquinolone resistance is significantly associated with point mutations in the *gyrA* gene which encodes the A subunit of DNA gyrase. However, it is not fully understood how particular mutations correspond with phenotypic resistance [199], [201].

3.1.8 Clarithromycin

Increasing rates of clarithromycin-resistant *H. pylori* is a major concern, due to the threat posed to public health. Resistance rates range from 14-17% in Africa, the Americas, and Southeast Asia, and are as high as 29-34% in other regions of the world. The

Maastricht VI/Florence consensus recommends that if local clarithromycin resistance rates are high (above 15%), clarithromycin should be avoided as a treatment option due to the increased likelihood of treatment failure [79]. Patients who have a strain of clarithromycin-resistant *H. pylori* are also significantly more likely to experience eradication failure when prescribed therapies containing clarithromycin. Secondary resistance is also generally very high in isolates from those who have failed at least one round of eradication therapy with clarithromycin, with resistance levels of 67% in the Western Pacific region [24]. Clarithromycin resistance is variable among age groups and between sexes, with females and patients over 60 both having a higher likelihood of harbouring a resistant strain [158]. However, a different study in the UK provided evidence that it is younger patients aged 21-45 that are more likely to have resistant strains [202]. Clarithromycin is a macrolide antibiotic (named for its structural macrocyclic ring (**Figure 12 E**)) that acts by binding the 23S rRNA component of the bacterial ribosome and inhibiting protein synthesis [181]. Resistance to clarithromycin is associated with single nucleotide polymorphisms on the 23S rRNA gene, which inhibit clarithromycin binding and allow the ribosome to continue functioning [203].

3.1.9 Rifabutin

Based on UKHSA guidelines, Rifabutin is recommended as a third-line antibiotic treatment for *H. pylori* infection, but after two previously failed eradication attempts, and on the advice of a specialist. Although there have been several studies that have shown the effectiveness of rifamycin-related compounds in the treatment of *H. pylori* infections [204], there are concerns that widespread use of the antibiotic could encourage the development of resistance. As rifamycin is one of the frontline treatments for tuberculosis (TB), an increase in the development of multidrug resistance would be a serious concern [205]. Rifabutin has instead been suggested for use in the case of rescue therapy after other treatment options have been tried and exhausted [206]. Studies have shown high eradication rates (80-95%) when included in triple therapies after one or more previously failed treatments, even against multi-drug-resistant *H. pylori* strains [204], [207].

Few studies focus on rifabutin resistance directly in *H. pylori*, with many instead using the antibiotic rifampicin (another rifamycin derivative) in its place. It was previously thought that because rifampicin and rifabutin are both structurally related antibiotics that target the same molecule, rifampicin resistance rates would be strongly indicative of rifabutin resistance rates [208]. However, a recent study has shown that the cross-resistance between

rifamycin derivatives is not robust, and it is inappropriate to use rifampicin to try and predict rifabutin resistance levels [209]. Therefore, although antibiotic susceptibility testing was performed on the Nottingham Strain Collection using rifampicin E-Test strips and disc diffusion methods, these results have not been included.

Fewer studies concerning rifabutin resistance in *H. pylori* have been published compared to the first- and second-line antibiotics, and it was not included in the 2022 WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) report [210], however, there is some available data. A 2009 study in Japan examined resistance rates of rifabutin in patients who had and had not previously been exposed to rifampicin. All seven out of seven isolates taken from patients with past rifampicin treatment demonstrated rifabutin resistance, while the isolates that were taken from patients who had not received prior treatment remained low [208].

In general, rifabutin resistance is low in populations with low previous exposure rates. A study in Dublin, Ireland with isolates collected between 2008-2009 found no resistance to rifabutin [211]. In Australia, a study examining the potential of rifabutin as a rescue therapy surveyed resistance rates in isolates from patients with previous clarithromycin-based treatment failure and found no resistance to rifabutin [204].

Rifamycin derivatives function by inhibiting the beta-subunit of the DNA-dependant RNA polymerase enzyme within *H. pylori*. They do this by forming hydrogen bonds with amino acid residues on the enzyme via the four hydroxyl groups on the ansa bridge and napthol ring (**Figure 12 F**) [212]. Point mutations in the *rpoB* gene which encodes this beta-subunit are significantly associated with resistance [213].

Rifabutin antibiotic discs and E-Test strips are not as widely available. Time and cost constraints prevented the re-testing of strains using rifabutin from being carried out, however, this may be within the scope of future work.

3.1.10 Resistance Rates in the UK

In the UK, resistance data has been measured sporadically in various locations, but continuous, wide-reaching data for all first- and second-line treatments have not been recorded. Of the studies that have been performed, it is apparent that resistance rates between regions and over time are variable, although it should be kept in mind that variations between methods of testing may in some part account for this, and in some instances there are different standards for accepted thresholds of resistance and susceptibility between studies [162].

In Suffolk in 1991-2001, metronidazole resistance was measured at 31.7% overall, with an increase from 29.1% to 37% over the course of the study. It was also found more commonly in women and young patients. Clarithromycin resistance was found in 5.3% of strains tested, however unusually, clarithromycin resistance was found to decrease over the study period from 10.3% to 3.8% [214]. A study of patients in different socio-economic groups in Sheffield between 1994-1999 found that 40.3% of samples displayed metronidazole resistance (increased from 20.8% over the course of the study), and that patients who were younger and female had a higher chance of harbouring a resistant strain, however, there was no apparent association between resistance rates and socio-economic status. There was no amoxicillin resistance apparent over the course of the study, but an increase in each for macrolide (erythromycin) resistance (0-5.8%), as well as tetracycline resistance (0-1.2%). There also seemed to be an association between metronidazole resistance and macrolide resistance [215].

Meanwhile, in London in 1994, a study of patients who had previously received nitroimidazole treatment found that those who had previously received metronidazole or tinidazole were more likely to harbour a metronidazole resistant strain (84%), and

female patients were also more likely to have resistant strains compared to male patients (54% and 18% respectively). Although all samples were taken from patients in London, resistance rates were found to vary based on the patient's country of birth; with 90% resistance for those born in Bangladesh and 67% for those born in the United Kingdom [216].

In 1998, a multicentre European study tested metronidazole, clarithromycin, and amoxicillin resistance, and found that metronidazole resistance was again significantly higher in females and teenage patients. Resistance in Gloucester to the three tested antibiotics was found to be 28.9%, 1.3%, and 1.2% respectively [217]. Another study in London in 1999-2000 had 59% resistance in metronidazole and 11% resistance to clarithromycin, and 8% of the samples had resistance to both, however, no resistance was found to amoxicillin or tetracycline [218]. A study from Bangor, Wales, in 2000-2003 measured 24% resistance in metronidazole and 7% for clarithromycin, with 4% of the strains tested being resistant to both. This also showed that resistance rates doubled over the course of the study, but that no difference between gender or age and resistance levels was detected [219].

In 2000-2005, a joint study in North Wales and in Essex found that resistance rates for both clarithromycin and metronidazole were

significantly lower in Wales (8.3% and 28.6%) than in Essex (12.7% and 36.3%), but that resistance had increased over the course of the study in both areas and were higher in females and patients under 45 years of age [202]. Further samples taken between 2009 and 2010 in hospitals in Bangor, Gloucestershire, and London showed resistance rates of below 3% for amoxicillin, tetracycline, and rifampicin. However, clarithromycin resistance was considerably higher in London and Bangor (68% and 18% respectively) than in Gloucestershire (3%), with similar results also being true for metronidazole and levofloxacin resistance (43% and 88% vs 22% respectively for metronidazole and 17% and 13% vs 1% respectively for levofloxacin) [220].

This shows an increase over time for fluoroquinolone resistance when compared with another study from multiple centres across the UK from 2001-2006, which placed fluoroquinolone (ciprofloxacin) resistance at 7.5%, and rifampicin resistance at >1%. This was the earliest *H. pylori* resistance study for the aforementioned antibiotics in the UK. Of these isolates, 88% of strains collected from patients who had previously received a failed round of therapy were resistant to 2 antibiotics [221]. More recently, in London between 2016 and 2018, in 36 isolates of *H. pylori* from patients that had failed one round of eradication

therapy, 35 were metronidazole resistant, 26 to clarithromycin, 10 to levofloxacin, 1 to tetracycline, and 0 to amoxicillin [222]. Again in London in 2017, an NHS hospital tested a small number of samples and found that resistance levels were 0% for amoxicillin, 66% for clarithromycin, 89% for metronidazole, 44% for levofloxacin and 11% for doxycycline [223].

As can be seen, there is a paucity of data available for *H. pylori* resistance in the UK, and of that which is available, much of it comes from the same few regions, leaving large areas of the UK devoid of data for local rates of resistance. Despite this, it is possible to see from available data that resistance to metronidazole has been steadily increasing since the 1990s, and that there is a great regional disparity between highly populous cities and more rural areas. There also seems to be a wealth of evidence to suggest that metronidazole-resistant strains are more prevalent in younger patients, however, the reasons for this are unclear. Metronidazole resistance also appears to be more prevalent in females, which has been suggested to be the result of metronidazole being a commonly used treatment for the protozoan *Trichomonas vaginalis,* which causes urogenital infection [224]. The data strongly suggests that a failed round of therapy may lead to the development of resistant *H. pylori* strains, although strains

with primary resistance are still common. Clarithromycin and metronidazole resistance has also been on the increase, and strains that are resistant to both of these antibiotics strongly forecast that a treatment will fail [152].

There is little historical data on resistance rates for the other antibiotics used in first- and second-line treatments, but the studies that tested for them seem to indicate in general that resistance rates to tetracycline, rifampicin, and amoxicillin have remained below the 15% threshold in the UK. Levofloxacin resistance also appears to have been on the rise, increasing in London from 17% in 2009 to 44% in 2018.

3.1.11 Evaluating Antibiotic Susceptibility

3.1.11.1 Disc Diffusion

Several different methods have been developed for assessing antibiotic resistance in bacterial samples. The methods vary in cost, information availability, and suitability for different purposes. A commonly used method is disc diffusion, in which a small filter disc is impregnated with a set concentration of antibiotic. Bacteria in a solution are then spread across an agar plate and allowed to dry, and the antibiotic disc is placed in the centre of the growth

medium. During incubation, the antibiotic will diffuse out into the agar and create a concentration gradient. The bacteria will grow up to a certain circumference around the disc but may leave a zone of clearing nearer to the centre, where the antibiotic concentration is high enough to inhibit growth or kill the bacteria [225]. The diameter of this zone can be measured and compared to published 'breakpoints' of susceptibility and resistance. Antibiotic resistant isolates will be able to grow closer to the antibiotic disc compared to susceptible isolates. While this method is one of the cheaper options, there are several issues present.

The methodology used in preparing agar plates and incubation conditions must be exact between tests, as these can change antibiotic diffusion and bacterial growth [226]. The size and shape of the zone of clearing may leave room for uncertainty if the edges are faint, or if the circle is irregular in shape. This method also only provides categorical 'resistant' or 'susceptible' results and does not provide quantitative information such as the minimum inhibitory concentration. There may not always be a consensus on what the appropriate breakpoint is for a given antibiotic, and conflicting breakpoints may be published in different papers [227].

3.1.11.2 E-Test Strips

E-Test strips work on similar concepts to disc diffusion methods. A thin plastic strip is impregnated with a gradient of antibiotic concentration and placed on an agar plate inoculated with bacteria. The antibiotic diffuses out and inhibits bacterial growth up to a certain concentration, however, the E-Test strip will have markings along it indicating the concentration of antibiotic present at that section. The MIC can be determined by looking at the concentration at the point where the bacteria cease to grow, allowing quantitative information to be gathered. Susceptibility breakpoints are also more widely standardized, with two agencies in particular publishing breakpoints yearly based on up-to-date information: EUCAST in Europe, and the Clinical Laboratory Standards Institute (CLSI) in the United States. While they can be more informative than antibiotic discs, E-Test strips can be more expensive and difficult to obtain.

3.1.12 Broth and Agar Dilution Assay

While dilution assays can be time-consuming, the process can be automated to speed up the turnover of samples and reduce errors from manual measuring and handling. In broth dilution, growth media is dispensed into micro-centrifuge tubes or disposable 96-well microtiter plates. The media is prepared with a range of

antibiotic concentrations, and then inoculated with bacteria. After incubation, the tubes are checked for bacterial growth, identified by turbidity of the broth, with a clear media indicating that cell growth has been inhibited [225]. This can be determined either manually or by an automated system. Markers such as pH indicators may also be used depending on the bacteria being tested. The tube with the lowest antibiotic concentration in which bacteria have been unable to grow provides the value for the minimum inhibitory concentration [226].

Agar dilution assays work on a similar concept to broth dilution but require the preparation of a series of agar plates containing a range of concentrations of the antibiotic being tested. A bacterial suspension is spread across the plates, and the plate with the lowest concentration of antibiotic that remains clear of bacterial growth provides the MIC value. Agar dilution is considered by some to be the 'gold standard' for determining MIC values [228].

Dilution assays can be expensive and can take up a large amount of laboratory space and reagents if high numbers of isolates need testing. Automation has a high initial setup but may save cost in the long run and can automatically process samples overnight to provide a quicker turnover of results. Care must be taken to avoid contamination, and to be sure that turbidity in the media is due to

the presence of viable cells only, so a level of expertise is required for this method [227].

3.1.13 Comparison of Suitability for Antibiotic Susceptibility Tests

All of the above methods of antibiotic susceptibility testing have uses under different circumstances. However, care must be taken when deciding which method is most suitable for a given set of circumstances. All of them can provide general information on antibiotic susceptibility, however considerations such as cost and time should be taken into account. Adequate quality control is also needed when using any testing methods to ensure consistent results, and knowledge of the effects of the antibiotics on the bacteria. For instance, antibiotics that have bacteriostatic rather than bactericidal effects need to be considered during testing, as bacteriostatic drugs may leave viable bacteria behind [228]. Some countries may also require particular methods or breakpoint types to be used for the sake of standardisation. For example, the CLSI only accepted the agar dilution method for antibiotic susceptibility testing. However, the accuracy and accessibility of antibiotic susceptibility tests should also be considered.

When comparing or compiling resistance data, the testing methodology should also be taken into account. E-tests have been

noted to have a higher degree of alignment with agar dilution results compared to disc diffusion [229]. Comparative testing in Indonesia between E-test and agar dilution methods showed acceptable levels of MIC agreement (>90%) between metronidazole, levofloxacin and clarithromycin, but with only fair agreement for amoxicillin. Despite this, metronidazole had high rates of 'very major errors' which is defined as the test returning a 'sensitive' MIC result for the sample when tested via E-test, whereas the gold standard agar dilution returned a 'resistant' result [230]. Another study in China found that disc diffusion and E-test results were 100% comparable for levofloxacin [165], whereas metronidazole has been found to have poor comparability [231]. Due to this, additional testing was performed to verify our metronidazole disc diffusion data, and any results from disc diffusion testing that had an 'intermediate' breakpoint, or in which the repeats gave variable results were repeated using E-tests.

3.1.14 Changing Use of Breakpoints

Previous publications of EUCAST guidelines for E-tests also contained intermediate results for clarithromycin. Up until the 2022 version of the guidelines, a resistant MIC was given as > 0.5 while a susceptible MIC was given as ≤ 0.25, which meant that MIC values between those figures were intermediate, leaving room for

misinterpretation of results. In the 2023 version of the figures, the clarithromycin resistance breakpoints were changed to resistant > 0.25 and susceptible ≤ 0.25 [232].

There are no universally accepted breakpoints for disc diffusion. For instance, several different papers have published breakpoints that define resistance as values that range from 11 to 20mm for a 5µg metronidazole disc [233]. However, there are also differences in methodology and MIC information available between EUCAST and CLSI, with different organisms present and different breakpoints provided for organisms that were covered in both sets of guidelines [234]. Breakpoints are published yearly and may change over time which is something to be aware of when assessing data from older publications. Additionally, there is a cost associated with purchasing the yearly CLSI updated breakpoint tables, while EUCAST publishes their breakpoints online with free access. This may influence which guidelines are used particularly in laboratories with limited funding.

EUCASTs antibiotic resistance breakpoints are decided by a committee, and knowledge of the bacterial resistance mechanisms and the pharmacokinetic properties of the antibiotic are taken into account, which is why the breakpoints are not consistent across different antibiotic agents for the same bacteria [235]. Each of these methods require the isolation and culturing of bacterial

samples, which particularly in the case of *H. pylori* can delay the process of obtaining results due to long growth times.

3.1.15 Aims and Hypotheses

The aim of this chapter is to assess the antibiotic resistance profiles of clinical *H. pylori* isolates from the Nottingham Strain Collection to 5 antibiotics (amoxicillin, tetracycline, clarithromycin, levofloxacin, and metronidazole). Using this data, and additional patient and genotypic data previously obtained, there are several hypotheses that can be tested concerning the relationship of antibiotic resistance and a variety of factors. As such, the hypotheses for this chapter are as follows:

1. In agreement with many studies from around the world, the rates of clarithromycin and metronidazole resistance in *H. pylori* will be higher than resistance to the other antibiotics.

2. Infections with multiple strains are uncommon in the UK, and therefore the antibiotic resistance profiles of isolates collected from the antrum of a patient will match the resistance profile of isolates collected from the corpus of that same patient.

3. In consonance with published literature from worldwide studies, the frequency of patients colonised by

antibiotic-resistant strains of *H. pylori* will have increased over time.

4. As failed eradication is a driver for the acquisition of secondary antimicrobial resistance, where patients have previously had a failed round of *H. pylori* eradication therapy, the frequency of antibiotic resistance will be higher than in patients who have not.

5. Since inflammation is a driver for DNA damage and mutation, antimicrobial-resistant strains will be more common amongst patients colonised with more virulent strains *i.e. vacA* i1 and *cagA*+ strains.

3.2 Methods

3.2.1 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was conducted on 241 *H. pylori* isolates gathered from 162 patients. Media and isolate preparation were performed as described in the General Methods section (**Chapter 2: General Materials and Methods).** Initially, disc diffusion susceptibility testing was performed using amoxicillin, clarithromycin, rifampicin, tetracycline, levofloxacin and metronidazole. E-Test strips were used later when available for all remaining isolates, and any isolates that had resulted in unclear or inconclusive susceptibility data were repeated using E-Tests as

well. To ensure that pooling of the E-Test and disc diffusion data would not be problematic, verification of results for a subset of isolates was performed as described below.

3.2.2 Comparison of Disc Diffusion and E-Test Methods

A test was performed to verify whether results for E-test and disc diffusion antibiotic susceptibility profiles were concordant with one another for the same isolates. To do this, 30 isolates were selected that had a range of both resistant and susceptible MICs. Susceptibility testing was performed on these using both methods, for five of the six antibiotics. Tetracycline was excluded as no resistant isolates from the Nottingham Strain Collection had been identified that could be used for verification purposes. Furthermore, metronidazole has an additional category, 'intermediate resistance', that was a possible result from using the disc diffusion method. An additional 16 isolates were therefore tested using metronidazole, in order to compare the two testing methods with a higher degree of confidence. Later published research found that rifampicin resistance was not comparable to rifamycin resistance. As rifampicin is not used in therapies for *H. pylori* those results are irrelevant and were not included [209].

3.2.3 Statistical Analysis

Data was collected and recorded in Microsoft Excel. Statistical analysis was performed using GraphPad Prism 9.5.1 software. A Fisher's exact test was used to analyse frequencies of resistance between patients who had or had not previously undergone eradication therapy. For analysis of antibiotic resistance by year, a Chi-square test for a trend was used. P-values of <0.05 were considered statistically significant.

3.3 Results

3.3.1 Isolates Tested for Antimicrobial Susceptibility

Of the 162 infected patients in the study, 63 had duodenal ulceration, 18 had gastric ulceration and 1 had gastric cancer. Forty-one patients in the study had previously undergone rounds of *H. pylori* eradication therapy and, upon return for further investigation, were still found to be *H. pylori*-positive. The number of previous rounds of therapy was not always specified in the endoscopy notes but, where this information was provided, the highest number was five. 121 of the patients were infected with a more virulent cagA+ strain, and 102 patients had *H. pylori* of the more virulent *vacA* i1 type.

Gastric biopsies were collected from the antrum and corpus regions of each patient's stomach during endoscopy. Isolates were cultured from both antral and corpus biopsies from 79 patients. 46 patients yielded an isolate only from the antrum, and isolates were recovered only from the corpus of a further 37 patients. This yielded a total of 241 isolates for characterisation; 83 were single isolates from individual patients, and 158 were paired isolates from the antrum and corpus. The latter enabled the investigation of whether the isolates from the two gastric regions of the same patient had different antibiotic sensitivities.

For the isolates tested using E-Test strips, the mean values and total range of MICs are summarized in (**Table 4**). When isolates grew immediately adjacent to the E-Test strip, with no zone of clearance, the MIC was recorded as the highest concentration on the test strip (32 μg/ml for levofloxacin, 256 μg/ml for all others). This was the case for 15/19 clarithromycin-resistant isolates, all 10 levofloxacin-resistant isolates, and 26/45 metronidazole-resistant isolates. Of the six amoxicillin-resistant isolates, the MICs ranged between 0.19 and 0.75 μg/ml.

Table 4 *- Summary of MIC data from E-Tests*

	Clarithromycin	**Amoxicillin**	**Tetracycline**	**Levofloxacin**	**Metronidazole**
Mean MIC (µg/ml)	80.0	0.055	0.118	5.1	60.9
Range (µg/ml) (minimum and maximum values)	0.016 to >256	0.016 to 0.75	0.016 to 0.565	0.036 to >32	0.016 to >256
Number of isolates tested	59	100	58	63	126
Resistant/sensitive	23/36	6/94	0/58	9/54	45/81

3.3.2 Comparison of E-Test and Disc Diffusion Methods

Because the E-Test method is more expensive than the previously used antibiotic disc diffusion protocol, we looked to develop the disc method and assess the results against E-Tests. For this, 30 isolates were tested side by side, and the categorical results (resistant, sensitive and intermediate) were compared (**Table 5**). The results showed 100% agreement between the tests for clarithromycin (20 resistant, 10 sensitive), amoxicillin (4 resistant, 26 sensitive) and levofloxacin (6 resistant, 24 sensitive). When testing metronidazole susceptibility using disc tests, there is the complicating possibility for an intermediate result, therefore an additional 16 isolates were tested, and the results were compared.

Table 5 *- Comparison of results from disc diffusion and E-Test assays.*

Antibiotic	Test method	Resistant	Sensitive	Intermediate
Clarithromycin	**Disc**	n=20	n=10	N/A
	E-Test	n=20 mean MIC 174.8 µg/ml (range 1.5 to >256 µg/ml)	n=10 mean MIC 0.027 µg/ml (range 0.016 to 0.094 µg/ml)	N/A
Amoxicillin	**Disc**	n=4	n=26	N/A
	E-Test	n=4 mean MIC 0.46 µg/ml (range 0.19 to 0.75 µg/ml)	n=26 mean MIC 0.036 µg/ml (range 0.016 to 0.125 µg/ml)	N/A
Levofloxacin	**Disc**	n=6	n=24	N/A
	E-Test	n=6 mean MIC >256 µg/ml (all >256 µg/ml)	n=24 mean MIC 0.207 µg/ml (range 0.064 to 0.19 µg/ml)	N/A
Metronidazole	**Disc**	n=36	n=6	n=4
	E-Test	n=25 mean MIC 198.56 µg/ml (range 12 to >256 µg/ml)	n=21 mean MIC 0.292 µg/ml (range 0.016 to 0.5 µg/ml)	N/A

N/A = not applicable

There were 36 resistant, 4 intermediate and 6 sensitive results from metronidazole disc diffusion tests, compared with 25 resistant and 21 sensitive results using E-Tests. Where the disc test indicated that the isolate was sensitive to metronidazole (zone of inhibition >21 mm; n=6), this result was completely replicated by the E-Test (mean MIC 0.31 µg/ml, range 0.016 to 0.5 µg/ml). As

anticipated, where there was no clearance around the antibiotic discs (n=22), the E-Tests provided the same resistant determination (mean MIC 213.5 μg/ml, range 12 to >256 μg/ml). Problems arose, however, where the diameters of visible zones were smaller than the breakpoint of 21 mm. In the four isolates with intermediate resistance from disc tests (diameters of 17 to 20 mm), these were all found to be sensitive by E-Tests (mean MIC 0.29 μg/ml, range 0.016 to 0.5 μg/ml). The disc tests were also less accurate in determining the susceptibility to metronidazole, when the zone diameters were 5-15 mm (14 resistant by disc diffusion; 3 resistant and 11 sensitive by E-Test). This meant that the disc diffusion test results were unreliable and the isolates with zones in this range had to be re-tested using E-Tests.

The remaining isolates were then tested using the disc diffusion method, or E-Tests as appropriate, to generate a full dataset totalling 241.

3.3.3 Antimicrobial Resistance Profiles of 241 *H. pylori* Isolates to Five Commonly Used Antibiotics

Of the whole collection of isolates, 31.9% were not resistant to any of the five antibiotics. 61.8% were resistant to metronidazole, 27.8% were resistant to clarithromycin and 2.5% were resistant to amoxicillin (**Table 6**). These are the commonly used antibiotics in

first line therapies in the UK. If the first round of treatment fails, then other drugs may be used in second- and third-line therapies including levofloxacin and tetracycline. No isolates were found to be resistant to tetracycline, but 4.1% were resistant to levofloxacin. 40.7% were resistant to a single antibiotic, 24.1% were resistant to two antibiotics (the vast majority of these being metronidazole and clarithromycin), and 3.3% were resistant to three antibiotics (clarithromycin, amoxicillin and metronidazole, or clarithromycin, levofloxacin and metronidazole, or clarithromycin, amoxicillin and levofloxacin). None of the isolates were resistant to more than three antibiotics tested.

The 8 isolates resistant to three antibiotics originated from 5 patients. Four had previously received eradication therapy, including one who had undergone 5 failed rounds of therapy (their paired antrum and corpus isolates were both resistant to clarithromycin, amoxicillin and metronidazole).

Table 6 *- Antimicrobial resistance data for all 241 isolates analysed in the study.*

Antibiotic	**Number of resistant isolates (from n=241), percentage**
Clarithromycin	67, 27.8%
Metronidazole	149, 61.8%
Levofloxacin	10, 4.1%
Amoxicillin	6, 2.5%
Tetracycline	0, 0%
Resistant to none of the antibiotics	77, 32.0%
Resistant to 1 antibiotic	98, 40.7%
Resistant to 2 antibiotics	58, 24.1%
Resistant to 3 antibiotics	8, 3.3%
Resistant to >3 antibiotics	0, 0%
Clarithromycin & metronidazole only	58, 24.1%
Levofloxacin & metronidazole only	5, 2.1%
Clarithromycin, amoxicillin & levofloxacin	2, 0.8%
Clarithromycin, levofloxacin & metronidazole	2, 0.8%
Clarithromycin, amoxicillin & metronidazole	4, 1.7%

Only the combination antibiotic resistance profiles that were detected are listed.

3.3.4 Resistance Profiles of the Paired Isolates

Of the 162 infected patients who donated gastric biopsies to the study, isolates were successfully cultured from both the antrum and the corpus tissues in 79 cases. Forty-six isolates were cultured only from the antral biopsies, and 37 isolates were cultured only from the corpus.

Of the 79 pairs of isolates, the antibiotic resistance profiles were identical in all but six. Two of these pairs differed in metronidazole resistance. In one instance the antral isolate was resistant with an MIC of 64 μg/ml, and the corpus isolate was sensitive (MIC 0.19 μg/ml). In another case the antral isolate was resistant to metronidazole (MIC 192 μg/ml) and clarithromycin (MIC >256 μg/ml), whilst the corpus isolate was sensitive to both antibiotics (MICs 0.047 and 0.5 μg/ml respectively). Three additional pairs differed in clarithromycin resistance, also with large differences in MICs (>256 and 0.016 μg/ml; 0.016 and 1.5 μg/ml; 0.38 and 0.125 μg/ml for antral and corpus isolates respectively). One pair differed in levofloxacin resistance (MICs of 0.25 and 12 μg/ml). Interestingly in one case, the isolate from the corpus biopsy was resistant to metronidazole (MIC >256 μg/ml), clarithromycin (MIC 1.5 μg/ml) and amoxicillin (MIC 0.75 μg/ml), whilst the antral isolate was only resistant to metronidazole (MIC >256 μg/ml). Of these six pairs of isolates, five had identical *vacA* i genotypes and

matching presence or absence of *cagA*. One pair differed in *cagA* status but were of the same *vacA* i1 type.

Apart from the mismatches in antimicrobial resistance between pairs of isolates from the same patient, different virulence factor genotypes were noted in a further five pairs. One pair had completely different *vacA* and *cagA* types (*vacA* i2, *cagA*+ in the antral isolate and *vacA* i1, *cagA*- in the corpus), three had the same *vacA* type but differed in *cagA* type, and one pair of isolates were *cagA* positive but had different *vacA* types. None of these had differences in their antimicrobial sensitivity profiles, however.

3.3.5 The Frequencies of Drug-Resistant Isolates from Patients With and Without Previous Eradication Therapy

To avoid bias from double counting the 79 pairs of isolates in the dataset, only one isolate per patient was considered further, and all data from the six discordant pairs were excluded. It was then determined for each individual patient (from the remaining total of 156) whether they harboured a drug-resistant isolate. We compared the proportions of patients with resistant isolates between two groups: 39 with a previously failed eradication therapy, and 117 for which there was no record of previous therapy to eradicate *H. pylori* (**Figure 13**).

23 of the 39 patients (59.0%) who previously received therapy were colonised with a clarithromycin-resistant isolate, compared with 19 of the 117 patients who had not (16.2%) (Fisher's Exact Test, p<0.0001). 33 of the 39 patients (84.6%) who previously received therapy harboured a metronidazole-resistant isolate, compared with 71 of the 117 patients who had not (60.7%) (p=0.006). Only 3 patients in total had amoxicillin-resistant strains, all having had previous therapy. Seven patients had levofloxacin-resistant isolates, 3 of whom had previously received therapy, and the difference was not statistically significant.

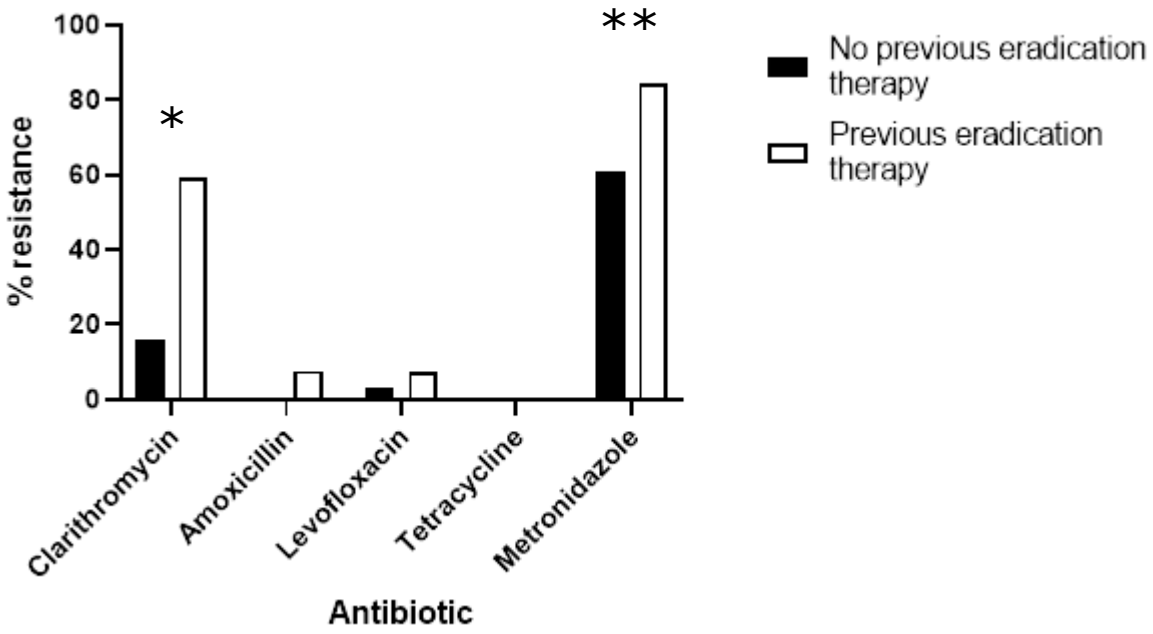


Figure 13 *- The frequencies of patients with resistant isolates, who had (n=39) or had not (n=117) previously undertaken H. pylori eradication therapy.*
The frequency of clarithromycin-resistant isolates was significantly higher amongst the patients with previous therapy (p<0.0001), and the same was true for strains resistant to metronidazole (p=0.006**).*

3.3.6 Antibiotic Resistance of *H. pylori* Isolates Over Time.

Trends in the incidence of antibiotic resistance were then examined over the 2001-2018 period of isolate collection, again based on whether the 156 patients harboured a resistant strain (**Figure 14**). The data were also broken down according to whether the patients had previously received *H. pylori* eradication therapy or not.

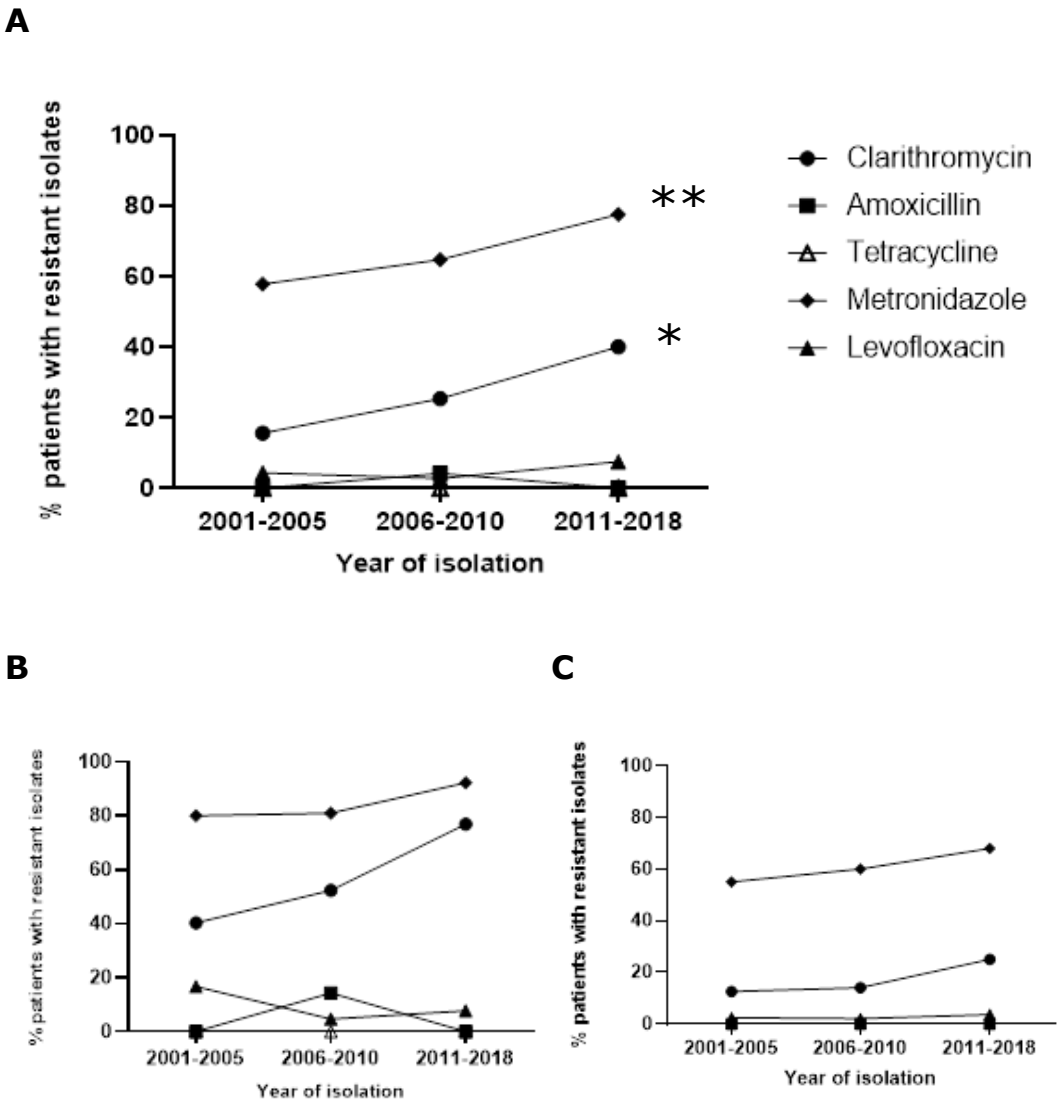


Figure 14 *- The frequencies of patients with resistant isolates, who had or had not previously undertaken H. pylori therapy over the period of the study.*

(A) The percentage of patients with resistant isolates from the entire dataset compared to the year the isolate was collected. 2001-2005 n=45, 2006-2011 n=71, and 2011-2018 n=40. (B) The percentage of patients with resistant isolates who had previously received eradication therapy, compared to the year the isolate was collected. 2001-2005 n=5, 2006-2011 n=21, and 2011-2018 n=13. (C) The percentage of patients with resistant isolates who had not previously received eradication therapy, compared to the year the isolate was collected. 2001-2005 n=40, 2006-2011 n=50, and 2011-2018 n=27. Significant trends in the data were found for clarithromycin-resistant (p=0.011) and metronidazole-resistant (p=0.05**) isolates, but only in the whole dataset (A). No significant trends were found for the other antibiotics, or within the subgroup analyses (B & C).*

In the whole dataset, from 156 patients (**Figure 14 A**), there was a significant increase in the frequency of patients with clarithromycin-resistant isolates from 15.6% (7/45) in 2001-2005 to 40.0% (16/40) in 2011-2018 (Chi-square test for trend, p=0.011). There was also a marked increase in metronidazole-resistant isolates, from 57.8% (26/45) in 2001-2005 to 77.5% in 2011-2018 (31/40) (trend p=0.05). When examining the data on patients who had previously received *H. pylori* eradication therapy (**Figure 14 B**), there was also an increase in the frequency of clarithromycin-resistant isolates from 40% (2/5) in 2001-2005 to 76.9% (10/13) in 2011-2018 but this did not achieve statistical significance. The frequency of patients with metronidazole-resistant isolates was 80.0% (4/5) in 2001-2005 and 92.3% (12/13) in 2011-2018, which was also not statistically significant. The frequencies of clarithromycin-resistant and metronidazole-resistant isolates from patients who had not previously received eradication therapy also followed the same trends (**Figure 14 C**) and increased during the period of the study. The differences were not statistically significant, however. In 2001-2005, the frequencies of clarithromycin-, metronidazole-, amoxicillin-, levofloxacin- and tetracycline-resistant resistant isolates from this sub-group were 12.5% (5/40), 55.0% (22/40), 0% (0/40), 2.4% (1/40), and 0% (0/40). In 2011-2018, the corresponding

frequencies were 25.9% (7/27), 70.4% (19/27), 0% (0/27), 3.7% (1/27) and 0% respectively.

3.3.7 The Frequencies of Antibiotic-Resistant Isolates with Differing Virulence Factor Type.

Since there is an increased risk of disease arising from infection with a more virulent strain of *H. pylori*, it was investigated whether there were any differences in the frequencies of antibiotic-resistant isolates amongst the *cagA* positive and negative groups. Isolates from the 162 patients were all PCR genotyped for presence and absence of the *cagA* gene, and this result was confirmed by testing for anti-CagA antibodies in samples of their serum.

Of the total 241 isolates in the study, 166 were *cagA* positive and 66 isolates were *cagA* negative. 9 isolates were removed from the analysis, as the PCR and serology results did not match. The proportions of antibiotic-resistant isolates in the *cagA* positive group were very similar to the *cagA* negative group (**Table 7**). There were no statistically significant differences.

Similar results were found when stratifying the isolates according to *vacA* i1 and i2 types (data not shown), where i1 strains are associated with increased risk of disease [68]. In addition, there were no significant differences in the frequencies of antibiotic-

resistant isolates from male or female patients, and there were no differences in isolates taken from tobacco smokers compared to non-smokers (not shown).

Table 7 *- Frequencies of antibiotic resistance amongst the cagA positive and negative isolates (n=232).*

	Number and percentage of antibiotic-resistant isolates				
	Clarithromycin	**Amoxicillin**	**Levofloxacin**	**Tetracycline**	**Metronidazole**
cagA+ (n=166)	43; 25.9%	5; 3.0%	4; 2.4%	0; 0%	100; 60.2%
cagA- (n=66)	20; 30.3%	0; 0%	2; 3.0%	0; 0%	43; 65.2%

3.4 Discussion

The success rate of standard triple therapy for eradication of *H. pylori* is decreasing in many parts of the world, where an increasing prevalence of antibiotic resistance has been found. The efficacies of regimens containing clarithromycin, levofloxacin and amoxicillin are seriously impacted by resistance to these antibiotics [236]. In order to ensure that treatment choices are appropriate and likely to be effective, it is important to investigate the antibiotic sensitivities of isolates in the local geographic region. These assays were performed on a collection of UK isolates, and a disc diffusion method was also developed that is reliable for clarithromycin, amoxicillin and levofloxacin susceptibility testing, which could provide others with a cheaper way to assess their isolates. This could overcome some cost barriers and facilitate better surveillance. The assay was not reliable for metronidazole tests, but this antibiotic can be an effective part of treatment regimens even when the strain is "resistant". Thus, the clinical utility of resistance testing for metronidazole is arguable.

In the collection of isolates gathered between 2001 and 2018, it was found that 32% were sensitive to all the main therapeutic antibiotics tested (clarithromycin, amoxicillin, metronidazole, levofloxacin and tetracycline). 41% of the isolates were resistant to one of these drugs, 24% were resistant to two, and 3% were

resistant to three. The prevalence of antibiotic resistance in our study has similarities to that reported by McNulty *et al.* in 2012 [220], based on isolates gathered in 2009-10 from three UK centres (London, Bangor and Gloucester). In our study we found 28% clarithromycin resistance overall, and 62% metronidazole resistance. For those without known previous *H. pylori* eradication therapy 16% were colonised by a clarithromycin-resistant strain. McNulty *et al.* [220] found higher rates of resistance to these first-line treatment antibiotics, at 68% and 88% respectively, in their isolates from London. The rates were lower (18% and 43%) in isolates from Bangor, and it was postulated that there was a higher frequency of resistant isolates in London due to more of the patients having been born outside the UK.

It is currently recommended that sampling is carried out to assess the prevalence of clarithromycin resistant strains [79], however currently no routine testing is carried out. It was detected that the frequency of clarithromycin-resistant isolates from patients without previous eradication therapy increased from 12.5% in 2001-5, to 14.0% in 2006-2010 and to 25.0% in 2011-2018. This is above the recommended 15% limit for its effective use as a first-line agent [79]. The observed trend is likely to increase further, as increasing global migration is resulting in the spread of *H. pylori* from countries with much higher frequencies of antibiotic resistance [237]. If clarithromycin is inappropriately prescribed for the

eradication of *H. pylori*, it greatly reduces the likelihood of treatment success, and unfortunately also increases the acquisition of resistance by other bacteria in the host microbiome [238], [239].

Metronidazole resistance was also more prevalent in the samples collected in 2011-2018, although the increase from the 2001-2005 sampling period was not as marked. Oral metronidazole accounts for just 1.8% of the total consumption of antibiotics in the UK, and its use has been declining in secondary care providers between 2017 and 2022 [240], although the COVID-19 pandemic accounted for reduced antibiotic usage across the board. Data gathered by the UK Health Security Agency show that since the COVID-19 lockdown has been lifted, there has been an increase in use for all antibiotic groups except metronidazole, which has had a decrease of 3.1% in usage between 2021 and 2022. Metronidazole was the second most prescribed antibiotic in dental settings, accounting for 28.2% of antibiotics prescribed by dentists during 2022 [241]. Oral antibiotics prescribed by dentists make up 10.8% of total antibiotics given by primary care practitioners in the UK, with amoxicillin being the most commonly prescribed (64.8%) between 2010-2017 [242].

An overall reduction in the prescription of metronidazole may indicate that the frequencies of resistant isolates are now

stabilising. A metronidazole-resistant isolate was detected in 78% of patients during 2011-2018, and in 92% of patients who had previously received eradication therapy during this time interval. Although these levels are high, resistance to metronidazole does not preclude its use in a therapeutic combination [243]. Higher doses of metronidazole can still be useful in treating *H. pylori* even in areas with high levels of antibiotic resistance, as an increased dose can partially overcome resistance [244]. Testing for metronidazole resistance is therefore of a lower clinical priority.

The frequency of amoxicillin resistance was found to be very low, with just 6 from a total of 241 isolates (2.5%). In line with our study, McNulty *et al.* [220] also found that amoxicillin resistance was rare at all three of their UK centres (London, Bangor and Gloucester). We were unable to find any tetracycline-resistant isolates, whereas the McNulty study found 4 isolates (1.7% overall). Other studies have also shown that tetracycline resistant isolates are very low or absent in the UK and in Europe. [215], [245]. It was found that the frequency of levofloxacin resistant isolates was also low (4%), but McNulty *et al.* reported 13% resistance in London, 17% in Bangor and 1% in the isolates from Gloucester. A study in France in 2018 reported 17.6% primary and 22.7% secondary resistance to levofloxacin [246]. A study of *H. pylori* antimicrobial resistance in 18 European countries in 2018 found that the frequency of primary levofloxacin resistance ranged

from zero in Denmark and the Netherlands, to 29.2% in Italy [245]. Use of levofloxacin is much less common in the UK than in other countries and has also been decreasing between 2017 and 2021 [240]. This may explain why we found fewer levofloxacin resistant isolates than reported for other countries outside the UK.

The data agreed with many other previous studies showing that a previous course of clarithromycin or metronidazole was associated with an increase in the risk of antibiotic resistance to that drug [220], [247], and significantly increased frequencies were also found of clarithromycin resistant and metronidazole resistant isolates over the time of sample collection [221], [247].

Some previous studies have identified that antibiotic resistance is more commonly found in *H. pylori* isolates from women, possibly because the nitroimidazoles family of antibiotics (which includes metronidazole) can be used in the treatment of gynaecological infections [248]. Metronidazole and clarithromycin resistance in Portugal has also been found to be more common in *H. pylori* isolates taken from women [203]. Women, and in particularly those who live in lower- and middle-income countries, are often more likely to be exposed to diseases (including antibiotic resistant bacteria) due to gender-based roles such as childcare, healthcare work and food preparation [249]. However, this study found no

identifiable differences in resistance rates between isolates from male and female patients.

It was also hypothesized that there would be higher levels of antimicrobial resistance in the isolates taken from patients who had a history of smoking. Smoking tobacco causes inflammation and can drive the development of antibiotic resistance, and additionally a US study found that smokers are 20-30% more likely to be prescribed antibiotics [250]. Smoking has also been shown to reduce treatment efficacy and lead to *H. pylori* eradication failure [251], [252]. Treatment failure is associated with the development of secondary resistance [253], and as such it was hypothesized that there would be higher levels of resistance found in isolates taken from patients who had a history of smoking tobacco. However, unlike some other reports, there were no associations with antibiotic resistance levels and cigarette smoking [192], [254].

It was surprising to find that six isolates (from four patients, three of whom previously had undergone eradication therapy) were resistant to amoxicillin. One isolate had an MIC of 0.19 μg/ml, close to the breakpoint concentration (0.125 μg/ml). Three had MICs of 0.5 μg/ml or greater. A recent study from Vietnam found a

frequency of 25.7% amoxicillin resistant isolates, which had an MIC range of 0.19–1.5 µg/ml. 44% of their resistant isolates yielded MICs of 0.5-1.5 µg/ml, which is extremely concerning [192]. *H. pylori* rarely possess a beta-lactamase gene, although it has been found in one instance [190]. Instead, it has been shown that the resistance phenotype was linked to seven amino acid changes in penicillin binding protein 1A. Work can be conducted in the future to analyse genome sequences of the amoxicillin-resistant isolates in the Nottingham Strain Collection to investigate mutations in their penicillin binding protein genes.

It was found that the vast majority of paired isolates, from the antrum and corpus tissues of the same patient, had identical antimicrobial resistance profiles. Six sets of isolates had different profiles, however, and one of these also differed in *cagA* status. This may indicate that some of these patients were infected with multiple strains. Presence of more than one strain could be a factor in the failure of eradication therapy, and it has been recommended that this possibility should be considered when carrying out antimicrobial susceptibility testing [55]. In the future, genomic analysis could be carried out on the discordant pairs of isolates to confirm whether there were multiple strain infections in these six patients. Previous studies have been carried out on the Nottingham Strain Collection, where whole-genome deep sequencing was performed on a small subset of the isolates described here (but not

the six described above), and it was found that there was extensive allelic diversity amongst populations within the antrum and corpus regions of each patient's stomach [56]. Antrum and corpus populations from the same patient grouped together in phylogenetic analyses, indicating that most patients were initially infected with a single strain, which then diversified. Recombination was observed both within and between different regions of the same patient's stomach. These findings were in agreement with Ailloud *et al.* (2021) [255], who found gastric region-specific diversity and recombination with bacteria from different regions. Their data also suggested that antibiotic treatments (including those for extra-gastric conditions) are likely to have a major influence on the population structure of *H. pylori* in the stomach. This is another potential driver of the global trend for increasing prevalence of antibiotic-resistant *H. pylori* [79].

The data presented here are important because there is currently very little antibiotic sensitivity testing of *H. pylori* isolates in the UK. This is despite guidance that *H. pylori* sensitivity testing should be carried out when patients have a restricted choice of antibiotic due to hypersensitivity, have previously received two unsuccessful courses of antibiotic treatment, or when known local resistance rates are high [5]. The main barriers to testing in the UK include the requirement for endoscopy to obtain isolates, the technical difficulties involved in culturing, and the associated costs. Data on

local resistance rates are unavailable for the UK and, considering the findings presented here, this puts patients at high risk of being given ineffective first line therapies. Multiple further rounds of therapy may be needed in order for the infection to be eradicated successfully, which could have detrimental effects on the patient including exposure to more antibiotic side effects, and the continuation of symptoms. It has recently been recommended that patients are tested for clarithromycin resistant *H. pylori* prior to prescribing this drug [79], however no such tests are routinely carried out in the UK at the present time. It is important that testing of *H. pylori* isolates should be improved in the UK, especially when considering clarithromycin as a therapeutic option. Stool-based PCR tests for clarithromycin resistance have been shown to be extremely accurate [256], [257]. In a recent study in France, comparing the Amplidiag *H. pylori*+ClariR stool PCR test for detecting resistance to clarithromycin with data from E-Tests, the sensitivity was 98.4% and the specificity was 100% [257]. Such non-invasive testing has the potential to dramatically enhance successful therapeutic choices.

3.4.1 Rifampicin

It was decided that rifampicin resistance results should not be grouped with the other resistance data, due to recent studies

showing that rifampicin resistance should not be used to infer resistance to the third-line antibiotic rifamycin. However, the results will be briefly included here as they can still provide some insight into antimicrobial resistance trends as a whole.

Out of all of the tested isolates, the total percentage resistant to rifampicin was 12.8% (31/241), with no significant change in resistance over time -only varying from 8% to 11% to 7% over the averages of the three time periods measured. Rifampicin resistance was highly involved in multi-drug resistance combinations, with 58% (18/31) of the isolates that were resistant to rifampicin also being resistant to one or more other antibiotic. Every isolate that was resistant to both clarithromycin and rifampicin was also resistant to metronidazole.

When comparing resistance levels for virulence factors, it was found that there was a higher percentage of resistance among isolates that displayed the VacA i2 isotype (14/79, 18%) compared to the VacA i1 isotype (14/152, 9%). Resistance levels were also higher in *cagA* positive isolates (25/166, 15%) compared to *cagA* negative ones (5/66, 8%), although these differences were not statistically significant.

There were also no significant differences found in resistance levels between males and females (13/18, 16% for both) and age group. The largest percentage of resistance isolates were found in the 51–

60-year-old age group, which contained 28% (8/29) of the resistant isolates. When comparing primary and secondary resistance levels, rifampicin was the only antibiotic that showed higher resistance levels in the group with no previous eradication therapy (24/120, 20%) compared to those who had a previously failed eradication therapy (6/31, 19%), however, this difference was not significant. There were 10 mismatched resistance profiles identified from pairs of isolates taken from the antrum and corpus of the same patient, the highest out of all the antibiotics tested (77 pairs tested). However, there was no clear partiality for the area of the stomach where the resistant isolate out of the pair would be located. Out of all the samples, 12.3% (14/114) resistant isolates were found in the corpus and 13.5% (17/126) were found in the antrum.

There were additional difficulties with using rifampicin disc diffusion in this study. It was found that this antibiotic in particular produced double zones or unclear edges to the zone of inhibition in some samples, making it more difficult to accurately measure and determine if it met the breakpoint size. The reason for these double zones or fuzzy edges could be that the colonies in the inner ring had reduced growth capabilities but the antibiotic concentration was not high enough to kill them. Additionally, the clinical isolates from biopsies are not selected for via individual colony picking, and so there may have been a mixed population of

resistant and susceptible bacteria within the samples, as multi-strain infections from this collection have previously been observed [56]. There are no specific EUCAST guidance for measuring rifampicin double zones in *H. pylori*, however, there is guidance for similar cases of bacteria that had fuzzy edge growth or double zones (**Figure 15**). The inner zone was measured from where the agar was completely clear, discounting any growth even of faint or very small colonies.

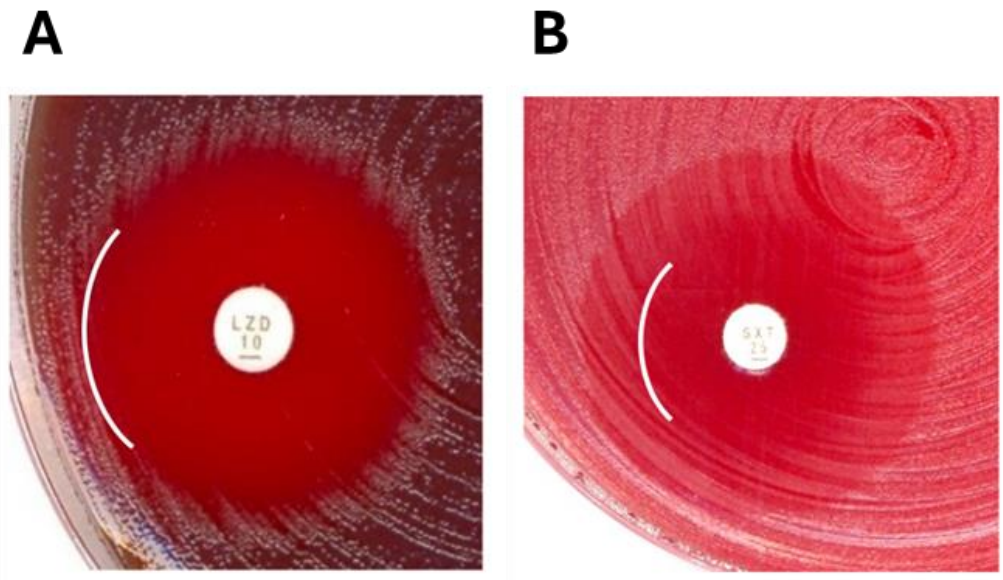


Figure 15 *- EUCAST Examples of how to measure*

A) fuzzy edge zones and B) double zones of inhibition. The diameter should be measured from the white curved line to the same place on the opposite side of the zone of clearing straight across the antibiotic disc. Taken from EUCAST Disc Diffusion – Reading Guide version 10.0 (2023) (Eucast: Disk Diffusion Methodology, 2023)

3.4.2 Future Work

If time and resources allow, there are several avenues that would be of interest for future exploration that would enhance the results of this study. Firstly, further collaboration would be needed between this research group and the gastroenterology team at Queens Medical Centre in Nottingham to continue the collection of biopsy samples. The most recent isolate was taken from a biopsy performed in 2018, and it is important that up-to-date resistance data is collected so that changing trends in antimicrobial resistance can be identified. If funds allowed, E-test strips should be used to perform the AMR testing on these samples, and any previous isolates that only have disc diffusion data should also be re-tested using E-tests so that MIC data is available for the entire collection. Additionally, it would be useful to obtain access to a patient's history of antibiotic use. Currently, there is only access to notes that detail if a patient has previously undergone *H. pylori* eradication therapy, and no information is available about which specific antibiotics were prescribed, and there is no data on whether any of the tested antibiotics were previously given to the patient for non-*H. pylori* related reasons. Access to this data with informed patient and doctor consent would provide valuable insight into the process of the development of secondary antibiotic resistance.

The data obtained so far has been from patients who were referred to the hospital for a biopsy, however patients who are asymptomatic or have mild symptoms are not represented. Given ethical approval and patient consent, *H. pylori* isolates could be cultured from patients who have tested positive but are not experiencing severe enough symptoms for hospital referral. This would help address any skew in the data that may be introduced by only testing bacteria that have caused more severe disease presentation. However, endoscopies are uncomfortable procedures with a risk inherent, and would require hospital appointments with specialists, who usually already have long waiting lists for procedures. To circumvent this problem, this group is researching potential alternative methods for collecting *H. pylori* samples for culturing, which do not require a pinch biopsy or an appointment with a specialist. One suggested method has been to develop a capsule connected to a string [259]. The capsule would contain a sponge or other material coated with a polymer that binds *H. pylori.* The patient would swallow the capsule which would dissolve in the stomach and possibly bind a bacterial sample, which could then be collected and used for culturing and antibiotic resistance testing.

3.4.3 Conclusion and Recommendations

In conclusion, although there was a trend of high and increasing resistance to clarithromycin and metronidazole, there was no resistance to tetracycline and the frequencies of amoxicillin and levofloxacin resistance were very low. The incidence of antibiotic resistance will probably have increased further since the 2018 isolate collection in our study, as observed in many other parts of the world [260], [261]. Patient antibiotic history should be strongly considered when prescribing therapies, to avoid the risk of treatment failure. The addition of bismuth into a treatment regimen should also be considered. The availability of *H. pylori* sensitivity testing in the UK should be expanded, including increased availability of stool-based PCR testing for clarithromycin resistance, to prevent unnecessary treatment failures and avoid exacerbating antimicrobial resistance in the gut microbiota.

Chapter 4: Phenotypic and Genotypic Levofloxacin Resistance in *H. pylori*

4.1 Introduction

Levofloxacin (also known by the brand name Levaquin) is a broad-spectrum antibiotic in the fluoroquinolone family and is effective against Gram-positive and Gram-negative bacteria. It is usually used as a treatment for tuberculosis, bacterial meningitis, and some bacterial pneumonia cases, although it is often only used as a last resort due to the rare possibility of severe side effects [262]. In UKHSA guidelines, levofloxacin is recommended as a second-line therapy against *H. pylori*, to be considered after an initial round of eradication therapy has failed, and if the patient has not had previous levofloxacin therapy for *H. pylori* eradication. The UKHSA guidelines state that levofloxacin should be given as part of a triple therapy along with a PPI as well as one other antibiotic: either metronidazole or amoxicillin depending on the patient's previous antibiotic exposure or penicillin allergy [5]. However, it has been suggested that levofloxacin-based triple therapy could be an effective and well tolerated alternative to clarithromycin-based first-line therapy, particularly in localities where clarithromycin resistance levels are high [263]. Resistance rates for levofloxacin are generally low worldwide based on the available data [24],

however, susceptibility testing in the UK is rarely carried out. Sequencing of isolates may be able to provide information on drug resistance more rapidly when deciding on treatment options for patients [264]. Identification of *H. pylori* antibiotic resistance via sequencing is beginning to see some use in diagnostic settings in the USA [265]. However, the concordance between levofloxacin phenotypic and genotypic resistance using PCR stool tests has been measured as 67% agreement [266], and further information about the role that levofloxacin resistance mutations play in treatment success would be of use.

4.1.1 Levofloxacin in General Clinical Use

Levofloxacin is a third-generation fluoroquinolone and was first patented in 1987. It has since found use in many healthcare applications due to its broad-spectrum action against enterobacteria, as well as its action against streptococci which first and second-generation fluoroquinolones were ineffective against [267]. Drugs in the fluoroquinolone family can usually be distinguished by the -floxacin suffix in the name. It is largely effective and has a low risk of severe side effects, so has been added to the World Health Organization's list of essential medicines, particularly for use against multi drug-resistant *Mycobacterium tuberculosis* [268]. Because of this important role

in the treatment of tuberculosis, the UKHSA has restricted the use of levofloxacin in some cases so that its continued functionality can be safeguarded. It can be used in the treatment of community-acquired pneumonia, soft tissue infection, and *H. pylori* infection but only as a second-line choice if a previous treatment has been ineffective [269]. In the USA, the Food and Drug Administration (FDA) has approved levofloxacin for a range of indications, including nosocomial and community-acquired pneumonia, skin infections, urinary tract infections, the plague (*Yersinia pestis*) and as a post-exposure preventative against anthrax [270].

4.1.2 Pharmacokinetics and Side Effects

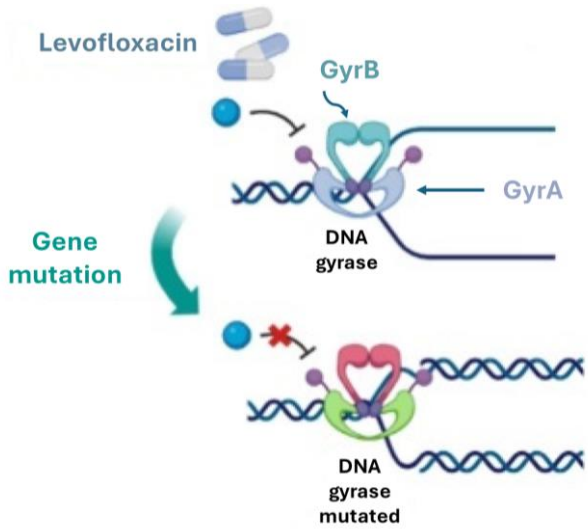
Levofloxacin is administered orally for the treatment of *H. pylori.* It is usually prescribed as 1 500mg daily dose or 2 daily 200mg doses [271]. It has a nearly 100% oral bioavailability, and plasma concentrations reach a mean peak of 5.2mg/L within ~2 hours of a 500mg dose and is not particularly affected by food consumption. It penetrates tissue and body fluids readily and is excreted in urine with a half-life of 6-8 hours, with 80% of the drug removed from the body unchanged [272]. Common side effects are usually mild and include nausea, disrupted sleeping, and diarrhoea (1-7%). More serious side effects include tendon inflammation and rupture (~0.03%), and peripheral nerve damage, as well as psychological

problems in rare cases (0.1-0.3%). Although the exact pathophysiology between levofloxacin and tendon rupture is not fully understood, it has been suggested that fluoroquinolones cause collagen degradation and necrosis of chondrocytes (collagen-producing cells) which damages the cartilage of the tendons, which in rare cases can cause them to rupture (mostly in patients over 60 or with previous tendon damage) [273]. Some of the rare neurological side effects have been linked to the fact that levofloxacin can inhibit GABA (gamma-amino-butyric acid) receptors, which stops the neurotransmitter from binding [274]. In some patients, this can lead to symptoms such as psychosis and schizophrenia [275]. Because of the impacts of some of the most serious side effects such as aortic aneurysm, in 2016 the FDA issued boxed warnings on the packaging of fluoroquinolones to more clearly inform patients of the risks [276]. However, for the most part fluoroquinolones are very well tolerated, and levofloxacin in particular has low risk of adverse effects, especially when balanced against the benefits of its use as a treatment for bacterial diseases [277].

4.1.3 Mechanisms of Action

Levofloxacin is a bactericidal antibiotic that functions by interfering with chromosomal replication during the cell cycle. Specifically, it inhibits the binding of two enzymes, Topoisomerase II and IV. Topoisomerase II (also called DNA gyrase) and Topoisomerase IV are two structurally similar but functionally distinct enzymes both used in DNA replication in bacteria. All enzymes in the topoisomerase group can relax the supercoils in the bacterial DNA chromosome to allow gene transcription and replication to occur (**Figure 16**). DNA gyrase is also capable of unknotting tangled DNA strands via strand passage, introducing negative supercoils into the DNA strand, which is an essential step in chromosomal condensation and relieving torsional strain during replication [278], [279]. The role of this enzyme in DNA replication means that DNA gyrase is an essential enzyme in bacterial species, but as it is not present in humans, it is a useful target for antibiotics [280].

A



B

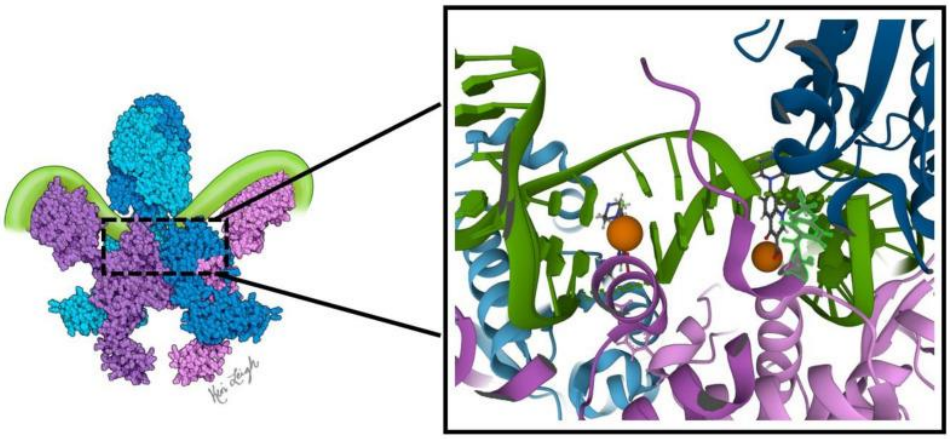


Figure 16 *- Structure and function of the enzyme DNA gyrase, including fluoroquinolone binding site.*

A) DNA gyrase comprises two GyrA and two GyrB subunits that combine into one structure. When functioning normally, DNA gyrase allows replication by relieving torsional strain on the double helix structure. Fluoroquinolones such as levofloxacin act by blocking DNA gyrase and halting DNA replication. Some mutations in the gyrA and gyrB genes can prevent levofloxacin from binding to DNA gyrase, making the bacteria resistant to its effects. Image taken from Spagnuolo et al [281]. B) Representation of a DNA/DNA gyrase complex, with a quinolone bound at the dimer interface. The green molecule represents a double-stranded DNA molecule, with the pink and purple structures representing two GyrA subunits and the blue and cyan molecules representing the two GyrB subunits. The two quinolone molecules are shown as ball-and-stick models, bound to Mg2+ ions shown in orange. Image taken from Spencer and Panda [282].

DNA gyrase is comprised of A and B subunits that join together in an A_2B_2 hetero-tetrameric structure. The subunits are encoded by the *gyrA* and *gyrB* genes found on the bacterial chromosome. The A subunits contain the active site that facilitates DNA binding and cleaving, while the B subunits are involved in the ATPase activity that drives the supercoiling [280]. Fluoroquinolone antibiotics, including levofloxacin, bind to the DNA/DNA gyrase complex and disrupt its action. Two molecules of the antibiotic can bind to the DNA gyrase hetero-tetramer at parallel points of the dimer interface, as shown in **Figure 16** [282]. Amino acids 87 and 91 form key binding sites for fluoroquinolones at the interface of GyrA, and as such they are sites of interest when identifying antibiotic resistance mutations.

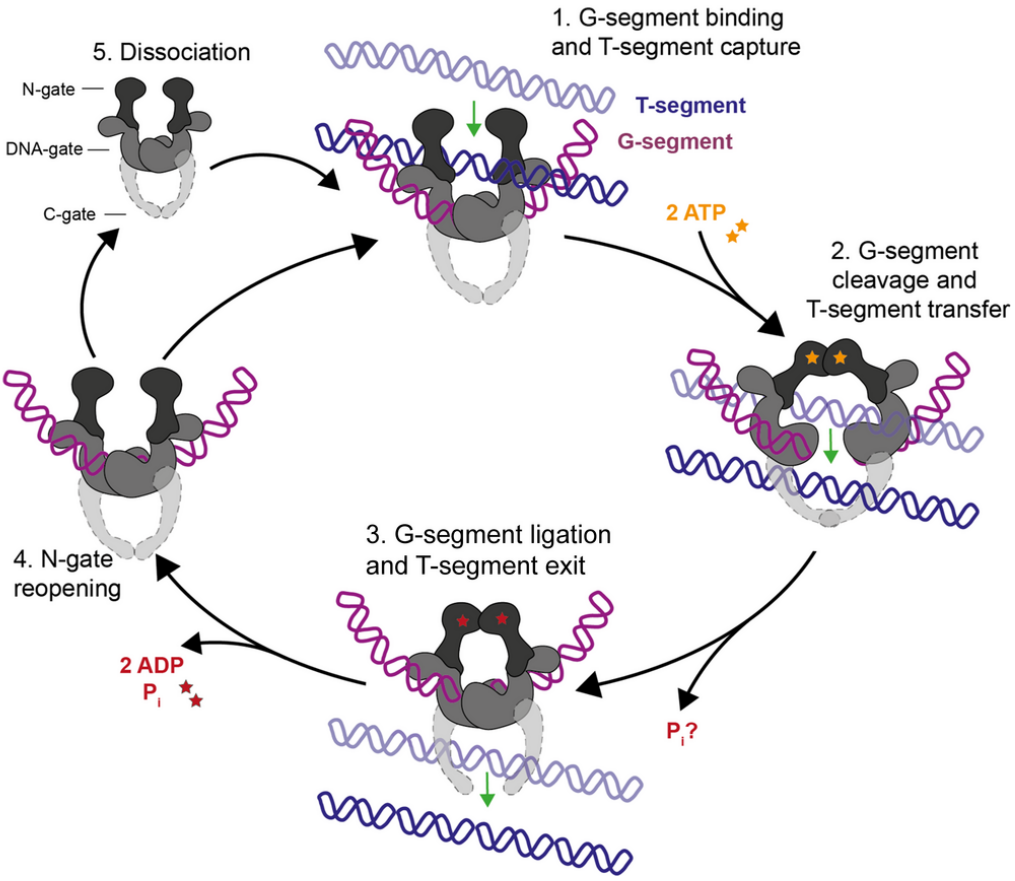


Figure 17 *- An example of the normal functionality of a Type II topoisomerase such as DNA gyrase.*

This image shows a pair of DNA strands; the blue transport segment (T-segment) and the purple gate segment (G-segment). The G-segment is bound to the DNA gyrase, and then ATP hydrolysis facilitates a conformational change which cleaves and separates the DNA strand. The T-segment DNA strand can then pass between the gap in the G-segment. The G-segment is then re-ligated and released, and the two DNA strands are separated. Image taken from Goedecke (2007) [283].

There is a greater wealth of structural biology research available for *E. coli* GyrA compared to the *H. pylori* version of the protein. However, due to their similarity in structure and characteristics, the details concerning *E. coli* GyrA can still be informative. When the enzyme is functioning properly, a tyrosine amino acid at *E. coli* GyrA position 124 (position 126 in *H. pylori*) forms a phosphotyrosine linkage with a DNA strand [284]. The DNA

binding interface of DNA gyrase will clamp and then cleave the double-stranded DNA molecule and temporarily separate the two ends into a 'gate' or G-segment. Usually, these two open ends would have a second unbroken double-stranded DNA molecule (the 'transport' or T-segment) passed through the newly made gap in a process called 'strand-passage'. Passing one section of DNA through another allows for several key processes such as introducing or removing coils and untangling knots in the DNA [279], [285]. The two disconnected ends of the G-segment are re-ligated, and the T-segment is released as shown in **Figure 17**. However, when a fluoroquinolone attaches to the DNA binding interface, the G-segment strands are still cleaved, but the strand passage and re-legation processes are blocked. Replication is inhibited, and the continued accrual of DNA breaks and torsional strain leads to cell death [286].

4.1.4 Mechanism of Resistance

The most predominantly studied and accepted mechanism by which *H. pylori* acquires levofloxacin resistance is via mutation of the *gyrA* gene. Fluoroquinolones bind to the GyrA enzyme at two specific sites, amino acids 87 and 91. A molecular docking experiment performed on *E. coli* GyrA shows that the two amino acids at positions 83 and 87 (which correspond to residues 87 and

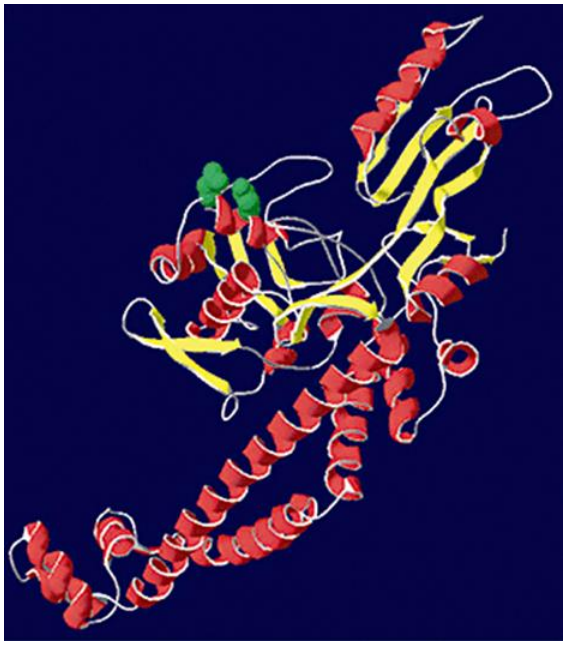
91 in *H. pylori*) form a water-metal ion bridge that links the antibiotic and the enzyme [287]. The 4 water molecules coordinate around a noncatalytic Mg^{2+} ion, and in this configuration, the water forms hydrogen bonds with the negatively charged carboxylate group of the aspartic acid at *H. pylori* position 91 [284]. The mutations commonly seen within resistant *H. pylori* strains do not have the same electrically charged side chain as aspartic acid, and as such the fluoroquinolone binding capability is reduced [287].

H. pylori can also employ several methods for resisting antibiotic treatment in addition to genetic mutations. Like many bacteria, *H. pylori* can form biofilms in which bacterial communities embed themselves into a matrix of extracellular proteins and polysaccharides [288]. This can help clumps of bacteria to adhere to host cells and prevent antibiotics from penetrating the film layer, which can increase resistance to levofloxacin by a factor of 16 [289]. Additionally, efflux pumps can reduce antibiotic susceptibility to a range of drugs. Efflux pumps are cell membrane transport proteins, which use ionic gradients or ATP to actively remove substances including antibiotics from a bacterial cell, and they have been found to significantly increase antibiotic resistance in *H. pylori*, including multi-drug resistance [290]. Inhibition of efflux pumps in *H. pylori* has been shown to restore antibiotic

susceptibility in multi drug resistant strains [289]. However, the mutations that cause the most widespread antibiotic resistance are caused by point mutations on the chromosome, instead of being acquired from plasmids or mobile genetic elements [291].

For levofloxacin, the majority of resistance genes identified in *H. pylori* have been mutations found on the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene. The QRDR section is considered to span amino acid residues 80 to 119 in the *gyrA* sequence [292], as shown in **Figure 18**. These mutations confer resistance to a range of fluoroquinolone antibiotics, as the mutations change the amino acid sequence of DNA gyrase subunit A and decrease the binding affinity of the antibiotics, reducing their efficacy. Mutations in the *gyrB* gene which encodes the DNA gyrase B subunit can also offer some protection against levofloxacin; however, these mutations are less commonly associated with a resistance phenotype compared to *gyrA* mutations [293].

A



B

Figure 18 *- The structure of GyrA and the location of the Quinolone Resistance Determining Region (QRDR).*
A) A protein homology model of a GyrA subunit. The green sections show the locations of amino acids 87 and 91, which are the most common sites of levofloxacin resistance mutations in the QRDR. Image taken from Matsuzaki et al., 2010 [294].
B) A schematic of H. pylori GyrA, with the QRDR (amino acids 67 to 106) highlighted in the red box. The blue arrows indicate residues 87, 91, 94, and 97, which are amino acid sites that have been found to have mutations linked to levofloxacin resistance. Black arrows indicate amino acid residues that are involved with levofloxacin binding (35, 87, 90, 91, 94 and 114-119). The pale orange highlighted areas are the catabolite activator protein (CAP)-like domain, which also contains the active tyrosine site (the single purple box), which in GyrA forms a transient covalent bond to the DNA strand during the cleaving action of DNA gyrase [295]. Light red highlighted sections are C-Terminal Domain (CTD) repeats, which function by wrapping and coiling the DNA that comes through the DNA gyrase gate. The blue section demonstrates where the GyrA-box motif is found within the CTDs. GyrA-box motifs are 7-residue sections conserved in gyrases that are essential for DNA supercoiling [296]. The green sections indicate the primary dimer interface, where the two GyrA subunits meet. Adopted from Salehi et al., (2019) [297].

Although the mutations associated with resistance are usually found on *gyrA,* there may be other resistance mechanisms at play [293]. There have previously been instances noted of *H. pylori* isolates where phenotypically the MIC was classed as 'susceptible', despite having *gyrA* mutations present that are significantly linked to resistance [298], [299]. Conversely, other studies have found strains that were phenotypically 'resistant' but did not appear to have any notable *gyrA* or *gyrB* mutations. Fluoroquinolone-resistant isolates in Cambodia [300], Korea [301], Japan [302], Poland [303] and China [199] have been sequenced, without any clear mechanism of resistance to be found. In some other bacterial strains, mutations in other chromosomal locations such as in the topoisomerase IV gene *parC* can confer resistance to fluoroquinolones, however, this gene is absent in *H. pylori* and so cannot be implicated [304]. Little research has been done on quantifying the fitness cost that levofloxacin resistance mutations may confer on *H. pylori*, but a study performed on another human digestive system pathogen *Arcobacter butzleri* showed that no significant fitness cost was found to be associated with *gyrA* mutations, whether that be in terms of motility or stress tolerance [305].

4.1.5 Amino Acid Substitutions and Resistance

The type and combination of mutation that occurs on the *gyrA* and *gyrB* genes can impact the fluoroquinolone MIC of *H. pylori*. Some SNPs that occur can be silent or synonymous mutations that do not change the amino acid encoded by the DNA, and as such do not have an impact on the resistance phenotype. The SNPs that do change the amino acid sequence may have more or less impact on the fluoroquinolone MIC based on the new amino acid. There have been several studies carried out that have attempted to elucidate the specifics of antibiotic resistance, and the types of SNPs present in DNA gyrase. The most common ones are single nucleotide polymorphisms, which change the amino acids present at positions 87 and 91 [306].

***Table 8** - Previously identified H. pylori gyrA mutations that have been associated with levofloxacin resistance.*

	Amino Acid Position				
Levofloxacin Status	**85**	**86**	**87**	**88**	**91**
Susceptible	Gly	Asp	Asn/Thr	Ala	Asp
Resistant	Cys (1)	Asn (2)	Lys (2)	Val (2)	Gly (4)
			Ile (2)	Pro (3)	Asn (4)
			Tyr (2)		Tyr (4)
					Ala (5)
					His (3)

(1) Rhie et al., 2020 [301], (2) Cattoir et al., 2007 [307], (3) Garcia et al., 2011 [308], (4) Nezami et al., 2019 [309] and Murakami et al., 2009 [310], (5) Wang et al., 2001 [311].

4.1.6 Amino Acids 87 and 91

In the *H. pylori* GyrA protein, the amino acid at position 87 in levofloxacin-susceptible strains is usually asparagine (Asn), however, there are also cases of susceptible isolates with threonine (Thr) in this position. Results published by Rimbara *et al*. (2012) showed that isolates with a lysine at position 87 can have a high levofloxacin resistance MIC of 16µg/mL, even when no mutation was present at position 91 [292]. Conversely, strains harbouring a Thr87 amino acid are more susceptible to some fluoroquinolones than the wild-type Asn87 variety. Rarely, isolates containing an Isoleucine (Ile) at position 87 have also been found, which provide

a high level of protection against fluoroquinolones with an MIC of 64μg/mL [307]. Murakami *et al*., (2009) found that the MIC range for mutations at position 87 was 4-32μg/mL, compared to 0.12-2 μg/mL for isolates without QRDR mutations. Compared to their measured levofloxacin resistance for mutations at position 91 alone (an MIC range of 2-8 μg/mL), it seems that mutations at position 87 produce high levels of antibiotic resistance in *H. pylori* isolates [310].

Interestingly, although a study into the binding of fluoroquinolones indicates that amino acid 91 in *H. pylori* plays a bigger role in drug/enzyme binding, it is changes at position 87 that tend to confer a higher resistance level [293], and as such there may be molecular binding interactions occurring at that site that are not yet fully understood [287]. This would also account in part for mutations at the flanking amino acid positions (86 and 88) that have also been linked to fluoroquinolone resistance, although less commonly. A mutation leading to an amino acid change at position 88 from alanine to valine (Val) has been found in ciprofloxacin-resistant isolates of *H. pylori* [312], and the same mutation was found in moxifloxacin and levofloxacin-resistant isolates as well [301]. An Ala88 substitution to proline (Pro) has also been identified in a ciprofloxacin-resistant isolate [308] (**Table 8**).

Concerning amino acid position 86, a mutation from Asp to Asn has been observed in fluoroquinolone-resistant isolates, although the role this mutation has in conferring resistance is unclear [307].

The *gyrA* 91 mutations usually substitute the wild-type aspartic acid (Asp) with a glycine (Gly), tyrosine (Tyr) or Asn residue [301], [313]. Additionally, a ciprofloxacin-resistant isolate containing a histidine (His) at this site has been cultured from a patient in France, although this mutation is rarer [308]. Of the above mutations, Asn91 substitutions seem to be the most common [303], and in general confers higher MIC levels (up to 32 μg/ml) than the other substitutions which tend to have an MIC range of 2-12 μg/ml, although there are exceptions to both sets of circumstances [292], [293], [308]. An alanine (Ala) substitution at position 91 that confers ciprofloxacin resistance has been identified via an *in vitro* spontaneous mutation assay, although this has not yet been seen in clinical isolates [311].

Generally, it is only a single mutation that confers antibiotic resistance to levofloxacin. However, cases where isolates contain mutations that lead to changes of both amino acid 87 and 91 have been shown in some studies to have greater fluoroquinolone resistance than those containing just one amino acid substitution

[293], [314], although other studies have failed to show this correlation [308]. It is thought that the combination of double mutations has an additive effect on MIC, although clinical strains containing these double mutations are rare which hampers further investigation.

The incidence rate of different amino acid substitutions may vary with geographical location of the strain, just as the overall *H. pylori* antibiotic resistance rate does [315]. Resistant isolates gathered in the cities of Paris and Poitiers in France were found to have the Thr87Ile substitution in 43% and 14% of samples respectively [308], and a comparison of *H. pylori* genomes originating from Europe and Asia indicated that the Asp91Asn substitution is more common in Europe [316]. These results indicate that certain resistance genotypes are linked to geographic regions, although there is a need for more data from different locations.

4.1.7 Additional Loci of Interest

Some regions of the *gyrA* gene have been flagged as potential sites of interest when trying to decipher antimicrobial resistance in *H. pylori* but have not commonly been observed in sequences from resistant isolates. For instance, molecular docking analyses performed by Chu *et al*., (2020) predicted that additional

mutations of interest may be found at amino acid positions 99 and 143, where substituting asparagine and glutamic acid (Glu) amino acids at these respective sites could lead to a weakened levofloxacin interaction [317]. In another study on *E. coli gyrA*, it has been found that Arg121 has a key role in fluoroquinolone binding. Although there have not been any definite links to resistance found in *H. pylori* clinical isolates at the corresponding site, it is a connection worth remaining cognizant of [287]. A paper published by Bińkowska *et al*., (2018) found that a codon triplet GCG (alanine) at position 122 of *gyrA* is significantly associated with levofloxacin resistance, although it is unclear why a GCG compared to the more commonly found GCA alanine triplet as found in *H. pylori* 11637 might cause resistance [303]. Some rarer, one-off mutations have been identified recently and may become more common in the future. A centre in Korea found a single case of a patient harbouring a levofloxacin-resistant strain containing a Gly85Cys substitution. A transformation assay confirmed that this mutation was responsible for the antibiotic resistance seen in the isolate [301], and in southern India, two levofloxacin-resistant strains had a five amino acid insertion directly after the start codon with the sequence QDNSV, with one also containing an Arg295His substitution [313].

4.1.8 Impact of GyrB Mutations on Fluoroquinolone Resistance

An additional contender for the cause of *H. pylori* fluoroquinolone resistance is the *gyrB* gene, which may offer some explanation for isolates that contain no *gyrA* mutations and yet still have a resistant phenotype. There have been some studies performed that have identified possible resistance mutations in this protein, but for the most part, it is in combination with the more common *gyrA* mutations. In a study in Japan in 2006 [293], 87% of the levofloxacin-resistant *H. pylori* isolates sequenced contained *gyrA* mutations leading to substitutions at either amino acid position 87 or 91, while only 3 of the 68 isolates (4%) had *gyrB* mutations. Of these three, two of them (Ile482Met and Glu463Gly) were associated with a GyrA 87 or 91 amino acid change, but one resistant strain had a GyrB Asp435Asn substitution and no associated *gyrA* mutations. Another study also found that a GyrB Glu463 substitution to Lys could confer norfloxacin and levofloxacin resistance in a transformed isolate without the presence of any *gyrA* mutations (**Figure 19**) [292]. Other papers have identified possible resistance substitutions at amino acid positions Ser457 [199], Ser479 [318] and Asp495 [301], however, these have all been in conjunction with GyrA 87 or 91 mutations. In *E. coli*, amino acids 426 and 447 (424 and 445 in *H. pylori*) have been associated

with quinolone resistance [292], although this has not been widely noted in *H. pylori* isolates. In general, it seems that *gyrB* substitutions are worth considering as having an impact on fluoroquinolone resistance, but it usually acts as a modifier for *gyrA* mutations with *gyrB* mutations independently conferring resistance far more infrequently.

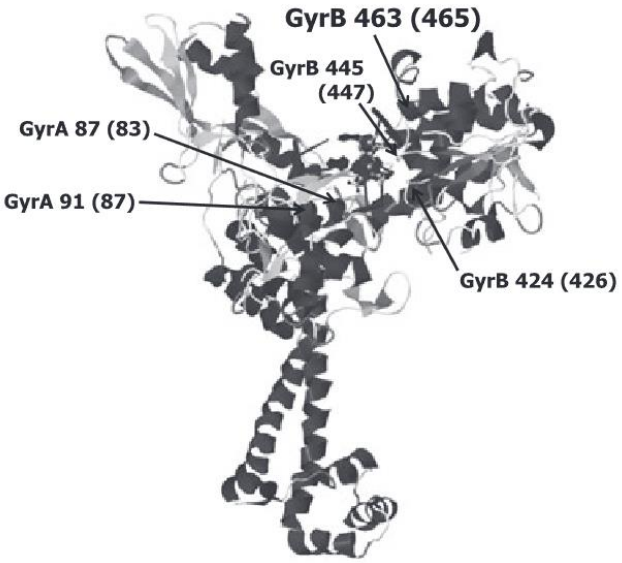


Figure 19 *– 3D structure of a GyrA and GyrB protein dimer, with common H. pylori fluoroquinolone resistance mutations labelled.*

Corresponding Escherichia coli positions shown in brackets. Image taken from Rimbara et al., 2012 [292].

4.1.9 Relationship Between Resistance Mutations and Treatment Success

While there is a link between *H. pylori* mutations and antibiotic resistance, the relationship between the number and type of mutations and the clinical treatment success should also be considered. How well genotypic resistance can be linked to treatment success is a very important question for researchers. A 2019 paper by Nezami *et al*., found no significant correlation between levofloxacin mutations and treatment failure, although only 3 patients in the study were treated with fluoroquinolones. However overall, the likelihood of treatment failure correlated with isolates that contained a higher number of mutations [309]. In 2011, Liou *et al*. found that patients who had *H. pylori* infections containing a *gyrA* mutation had eradication success rates of 41.7%, compared to an 82.7% eradication success rate for strains without *gyrA* mutations [306], meaning that the presence of mutations is a good predictor of treatment outcome.

Another study in 2012 looked more closely at the specific mutations present within *H. pylori* isolates from patients who had undergone a failed round of clarithromycin first-line and metronidazole-based second-line therapy. Isolates were obtained

from the patients, and then a third-line sitafloxacin therapy was given. The success rates of the fluoroquinolone-based therapy were compared to the *gyrA* mutations present in the isolates, and it was found that overall, treatment failure was more likely in patients harbouring a strain with a mutation at Asn87 (61.9%) compared to Asp91 (86.4%) [319], which aligns with similar findings for moxifloxacin [304] and levofloxacin treatments [293]. Asn87Lys was the most common mutation in the treatment failure group, and of the three Asp91 substitutions found in isolates within this study, only the Asp91Tyr variant was present in the treatment failure group. Interestingly, it was found that the presence of a mutation at *gyrA* 87 was a better predicter of treatment failure than a high antibiotic MIC [319].

A similar study by Mori *et al.* found that all the strains that did not contain any *gyrA* mutations were successfully eradicated with a triple therapy of PPI, amoxicillin and sitafloxacin, while eradication rates in groups containing any type of *gyrA* mutation had a significantly lower treatment success rate (70%). When comparing eradication rates for mutations at specific loci, it was found that position Asp91 had an eradication rate of 83.3%, but strains with mutations at Asn87 only had a 64% eradication success rate. Of these, the Asn87Lys substitution was the most common. This paper also showed that mutation rate, MIC and eradication rate were all significantly associated [320]. The same group later provided

further evidence that the sitafloxacin-based treatment eradication success in strains with an Asn87 mutation was significantly lower than for those with an Asp91 mutation (65% vs 82%) [302].

Taken together, these studies indicate that the identification of mutations at *gyrA* are a good predictor of treatment success when using a fluoroquinolone-based treatment. Both the quantity and type of *gyrA* mutation are linked to eradication therapy success and could be useful for informing patient treatment plans. However, there are still questions and considerations to be examined about the identification of these mutations, and the best methodology that could be used to facilitate better treatment options for patients.

4.1.10 Determining Resistance

As mentioned in previous chapters, resistance to fluoroquinolones is determined by phenotypic and genotypic methods [264]. However, in a clinical setting, *H. pylori* antibiotic resistance is rarely measured, and testing for resistance to first-line treatments like clarithromycin and metronidazole is of higher priority. With the significantly increased prevalence of *H. pylori* strains that are resistant to these first-line antibiotics (both globally and in Nottingham), alternative treatment plans need to be considered

[183]. If alternatives such as levofloxacin are implemented more commonly, procedures will need to be put into place to safeguard the continued effectiveness of the drugs and prevent the development of multi-drug resistance. As levofloxacin is used in the treatment of several serious conditions such as multi-drug-resistant tuberculosis, *Yersinia pestis*, and anthrax exposure [270], the development of resistance must be kept to a minimum. Ensuring appropriate treatment is prescribed is therefore essential. The gold standard of testing for antibiotic resistance is via phenotypic methods such as E-tests or agar dilution [228] (described in Chapter 3), however, genetic susceptibility testing is becoming more common [265], including the use of PCR-based tests [321].

4.1.10.1 PCR Testing

Polymerase chain reaction based tests are widely used for a huge range of applications including *H. pylori* diagnoses. In brief, PCR involves the amplification of a small section of DNA, so that very small quantities of genetic material can be duplicated many times over. The original DNA is heated to denature and separate the two strands from each other, and then the temperature is lowered again to allow short sequences of DNA called primers to anneal to the target sites of the two separated template DNA strands. A DNA

polymerase enzyme binds at the primer site and moves along the template strand one base at a time, adding the complementary bases (from free dNTPs added to the reaction) to the primer and extending the copy strand (**Figure 20**). Heating will once again separate these strands, and the process can repeat [322].

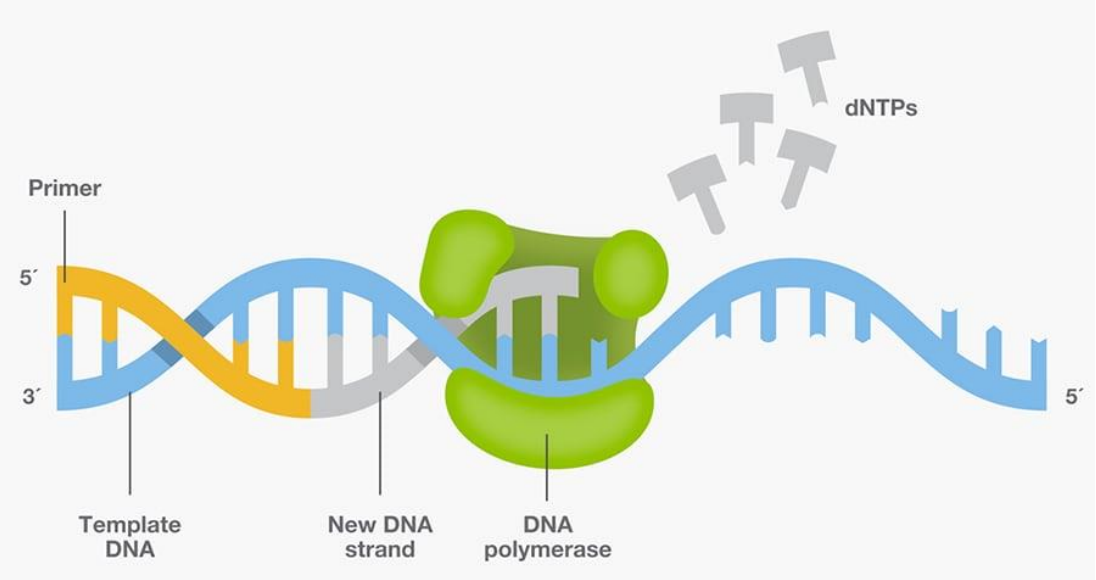


Figure 20 *- The process of amplifying a DNA sequence via PCR (polymerase chain reaction).*

The template DNA strand (shown in blue) is separated from the other template DNA strand by heating. A primer (shown in yellow) anneals to a section of the sequence and the DNA polymerase enzyme moves along the strand, adding the correct free nucleotides (dNTPs) and extending the new DNA strand. The template and new strand will separate upon heating, and the cycle will be repeated. Image taken from ThermoFisher Scientific [323].

This heating and cooling cycle is repeated multiple times (often 25-40), and each newly formed double-stranded section of DNA is replicated for as long as the reagents and thermocycling allow. The section of DNA to be amplified is defined by the primers used, which are short (~15-30bp) sequences that mark the start and end

of the segment of interest, such as a section of DNA that may contain an antibiotic resistance gene.

4.1.10.2 DNA Polymerase Enzymes

A vital component of the PCR reaction is the DNA-dependent DNA polymerase used. This enzyme moves along one template strand and builds up the complementary strand by adding the correct nucleotides that correspond to the template. Taq polymerases are selected because of their ability to withstand the high temperatures needed for separating the two DNA strands without themselves denaturing. However, there are several different types of polymerases available for use, which prioritise certain aspects of DNA replication over others (**Table 9**).

One of the options that was considered was DreamTaq DNA Polymerase (Thermo Scientific), which is a standard PCR reagent. It has an error rate of 2.2×10^{-5} per nucleotide per cycle, which is a measure of the number of incorrectly incorporated nucleotides per total number of incorporated nucleotides. The fidelity is the measurement of how accurately the bases in the template strand are replicated by the polymerase and is expressed as a comparison against standard *Taq* polymerase. DreamTaq has the same fidelity as *Taq* polymerase, and it can be easily substituted into *Taq* PCR protocols without needing to alter or optimise the thermocycler settings. However, compared to *Taq* polymerase, DreamTaq has

increased sensitivity and can yield fragments up to 6kb in size. The amplification performance of target DNA that has a high GC content is low, however, the GC content of the *gyrA* gene is 41.7% and so this is not applicable to our needs [324].

Table 9 *- A comparison of the replication properties of DreamTaq DNA Polymerase (Thermo Scientific) and Phusion High-Fidelity polymerase (New England Biolabs) for use in PCR.*

	DreamTaq	**High-Fidelity**
Fidelity (vs *Taq*)	1x	50x
Error Rate	$2.2x10^{-5}$	$0.44x10^{-6}$
GC Rich Performance	Low	High
Proof Reading	No	Yes

An alternative polymerase that was considered was the Phusion High-Fidelity polymerase PCR kit (New England Biolabs), which is designed to include fewer errors during replication. This would be of interest in this study due to the need to detect antibiotic resistance SNPs in the *gyrA* gene. Product specifications state that this polymerase has 50x higher fidelity than regular *Taq* polymerase, and a higher binding preference. The error rate is lower than DreamTaq at $0.44x10^{-6}$ errors per cycle, and there is also a 'proofreading' mechanism whereby if an incorrect nucleotide binds during replication, there is an additional exonuclease domain that the mis-paired base gets moved to and the most recently added nucleotide is removed. This gives an additional opportunity for the correct base to be inserted at that location. When attempting to identify single nucleotide polymorphisms that may confer antibiotic resistance, this may be an important factor,

however the higher cost of this polymerase may limit how useful it would be in clinical applications [325].

Amplifying a section of DNA via PCR is a relatively quick procedure, with results that can be returned the same day in comparison to the week needed to receive results from culture-based techniques. PCR tests only need a small amount of DNA to begin with, and they can be performed on stool or saliva samples which reduces patient time spent waiting for a specialist to perform a biopsy and culture the bacteria. Samples do not need specialist storage and transfer either, and the equipment needed for PCR testing is far more widely available than the microaerophilic incubators needed for culturing live *H. pylori* samples.

A drawback of sequencing-based resistance testing, however, is that only known resistance mutations will be identified, while novel mutations will not [326]. For PCR testing, resistance mutations outside the specific gene section amplified will be overlooked. Additionally, in the case of levofloxacin, there is still no consensus about how well the results of genotypic testing line up with phenotypic resistance. The practicality, accessibility and accuracy of SNP detection using PCR should also be considered, as well as how well this lines up with phenotypic resistance and treatment

outcome. These are considerations that still need to be reviewed before PCR testing can be widely implemented in a clinical setting in the treatment of levofloxacin-resistant *H. pylori*, and this study will look at addressing them.

4.1.11 Summary and Aims

The currently recommended first-line treatment for *H. pylori* is a clarithromycin-based triple therapy, however, local resistance rates are high, and an alternative should be considered. Levofloxacin is a potential alternative that has a comparatively low resistance rate and a good success rate for eradication therapy, however, safeguards are needed to maintain the effectiveness of levofloxacin and fluoroquinolones in general due to their importance in the treatment of other pathogens such as multi drug-resistant *M. tuberculosis*. Treatment failure when using levofloxacin-based therapies can lead to the development and spread of fluoroquinolone resistance in *H. pylori* and other bacterial diseases and is to be avoided. Prescribing effective medicines reduces treatment failure rate which is in the patient's best interest and constitutes good antimicrobial stewardship. While there is evidence that identifying mutations in the *gyrA* gene of *H. pylori* can help predict treatment success, there are still uncertainties regarding

exactly which mutations or combinations thereof will cause phenotypic resistance. Levofloxacin resistance genes are generally single nucleotide polymorphisms, and in order to detect them, a reliable and effective method should be employed. It is also important that this detection method be accessible and inexpensive so that it could be widely used in a clinical setting, while still being sensitive enough to identify individual mutations.

In order to address these problems, this chapter had several key aims:

1. **Select *H. pylori* isolates from the Nottingham Strain Collection, and assess antibiotic MICs from culture-based methods and investigate related gene sequences.**

Using results previously gathered from E-test susceptibility testing, all the levofloxacin-resistant *H. pylori* isolates were chosen for PCR amplification and sequencing. For comparisons sake, a selection of susceptible and borderline resistant isolates with a range of MICs were selected too. These isolates were then cultured, and the DNA extracted for PCR amplification.

2. **Perform PCR amplification of the *gyrA* gene and optimise the protocol for identification of mutations within the QRDR. Additionally, compare different polymerase enzymes for suitability of SNP detection.**

Amplification was performed using two different polymerase enzymes and a range of annealing temperatures. The amplicons were then sequenced, and compared to ascertain the most suitable reagents and methodologies for identifying single nucleotide polymorphisms related to antibiotic resistance.

3. **Identify *gyrA* mutations that may confer levofloxacin resistance and compare them with phenotypic resistance data with particular attention paid to mutations that occur in the QRDR.**

Amino acid substitutions were compared to the MIC data to investigate the relationships between particular mutations and resistance phenotypes.

4. **Assess potential benefits and drawbacks of gene sequencing for the identification of *H. pylori* levofloxacin resistance in a clinical setting.**

Consideration was made concerning how useful PCR product sequencing might be in a clinical setting, and how well PCR sequencing results correspond to treatment success. This may be used for informing treatment plans for patients harbouring antibiotic-resistant *H. pylori*.

4.2 Methods

4.2.1 Strain Selection

Isolates were selected from the Nottingham Strain Collection (see Chapters 2 and 3) that had an MIC indicating that they were resistant or borderline resistant to levofloxacin (LevR). Initially, nine strains that had an MIC of >1 (indicating that they were LevR) were selected, as well as 15 strains that were close to the breakpoint value of resistant, but still classed as susceptible (an MIC of between 0.25 and 1). Thirteen further isolates that had an MIC indicative of levofloxacin susceptibility were included as controls. This included paired antral and corpus isolates from the same patient, where one was classed as levofloxacin resistant and the other as susceptible (LevS). The laboratory strain *Helicobacter pylori* NCTC 11637 (DSM no: 21031) [327] was also sequenced for use as a control strain.

4.2.2 Determining levofloxacin MIC

As described in section 3.2.1, the levofloxacin MIC of each *H. pylori* isolate was determined [328] using levofloxacin E-test strips (BioMerieux, UK Ltd), and compared to the published EUCAST breakpoints [329] to determine if it could be classed as susceptible or resistant. This is detailed more fully in Chapter 2 and 3.

4.2.3 Strain Growth and DNA Extraction

Growth of the *H. pylori* isolates for DNA extraction was performed as described in Chapter 2, however, the number of 5% horse blood agar base no. 2 plates (OXOID, UK) used for the second passage was doubled to 4 for each strain, to provide as much bacterial growth as possible to increase the yield from the extraction. Bacterial growth from the 4 plates was then swabbed and suspended into 1mL of phosphate-buffered saline (PBS), and centrifuged at 16,000g for 5 minutes using an accuSpin Micro 17 (Fisher Scientific) benchtop centrifuge. The PBS was then discarded. Extraction of *H. pylori* genomic DNA was performed using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, product code NA2110-1KT), following the manufacturer's instructions.

Several steps were taken to ensure that an abundant yield of high molecular weight DNA could be procured. When resuspending the cells, 20µl of the RNase A solution provided in the GenElute™ kit was added and the mixture was incubated at room temperature for 2 minutes. Additionally, when the protocol instructed that the 1.5 ml Eppendorf tube be vortexed to mix, the tube containing the mixture was instead flicked to avoid degradation of the DNA.

For pipetting the extracted DNA, P1000 pipette tips were used that had been modified by cutting a small amount off the end with a pair of scissors before autoclaving. This was to provide a wide bore pipette tip to prevent mechanical shearing of the gDNA.

After washing the column containing the DNA, a second wash step was performed to remove as much ethanol as possible. Any residual ethanol was allowed to evaporate off the sample for 2 minutes before proceeding to the next step to avoid any contamination of the final product. Finally, the kit column containing the extracted DNA was incubated at room temperature for 5 minutes with 200µl Qiagen Buffer EB (Cat. ID 19086) to maximise elution efficiency. This elution buffer was used as it does not contain EDTA (ethylenediaminetetraacetic acid) which would interfere with downstream sequencing.

Once extracted, the gDNA was visualised via gel electrophoresis by mixing 5µl of the eluted DNA from each sample with 1µl of Gel Loading Dye Blue 6x (NEB, B70215), and running it on a 0.7% agarose gel (Bioline). DNA concentration was quantified using a SimpliNano™ NanoDrop (Biochrom), with a blank control reading performed using the same buffer that the samples were eluted with. The Nanodrop was also used to assess the quality of the DNA samples using absorbance ratios. Samples with an A_{260}/A_{280} absorbance ratio outside the 1.7-2.0 range were discarded due to

possible contamination and the DNA was re-extracted. Samples were stored in a -20°C freezer for short-term use.

4.2.4 Primer Design

A pair of PCR oligonucleotide primers were used in the amplification of the *H. pylori gyrA* gene. These were named GyrA-F (5’ GGCGTATTTTGTATGCGATGC) and GyrA-R (5’ TCAGGCCCTTTGACAAATTC). The sequence for the GyrA-F primer was taken from Zhong *et al.* (2021), and the GyrA-R primer sequence was taken from Wang *et al*., (2010) (**Table 10**), which together amplify a 510bp fragment of the Quinolone Resistance Determining Region of the *H. pylori gyrA* gene (**Figure 21**). This is the section of the gene where SNPs have previously been identified that are associated with levofloxacin resistance [293].

Table 10 - *Primers used for the amplification of a 510 base pair section of the gyrA gene of H. pylori.*

Primer Name	Sequence	Reference	Amplicon Size
GyrA-F	5’ GGCGTATTTTGTATGCGATGC	Zhong et al., (2021)	510bp
GyrA-R	5’ TCAGGCCCTTTGACAAATTC	Wang et al., (2010)	

Primers were analysed on NetPrimer (Biosoft International) to check for the likely formation of unwanted secondary structures and primer dimers. The PCR primers were ordered from MERCK (Sigma-Aldrich). A BLAST (Basic Local Alignment Search Tool) search of the *gyrA* sequence of the *H. pylori* laboratory strain Puno135 was performed to acquire the *gyrA* sequences from a range of different *H. pylori* strains. These were each opened in the software SnapGene® Viewer (Version 6.1.2), and the primer sequences were imported to assess if they would bind to the intended target sequences for a range of *H. pylori* strains even if they might have some mutations at the target site. Upon arrival, the primers were rehydrated in Tris EDTA (TE) buffer (Thermo Fisher Scientific) to a concentration of 100μM. A working stock of 25μM primer in nuclease-free water (NFW) was made and stored at -20°C between uses.

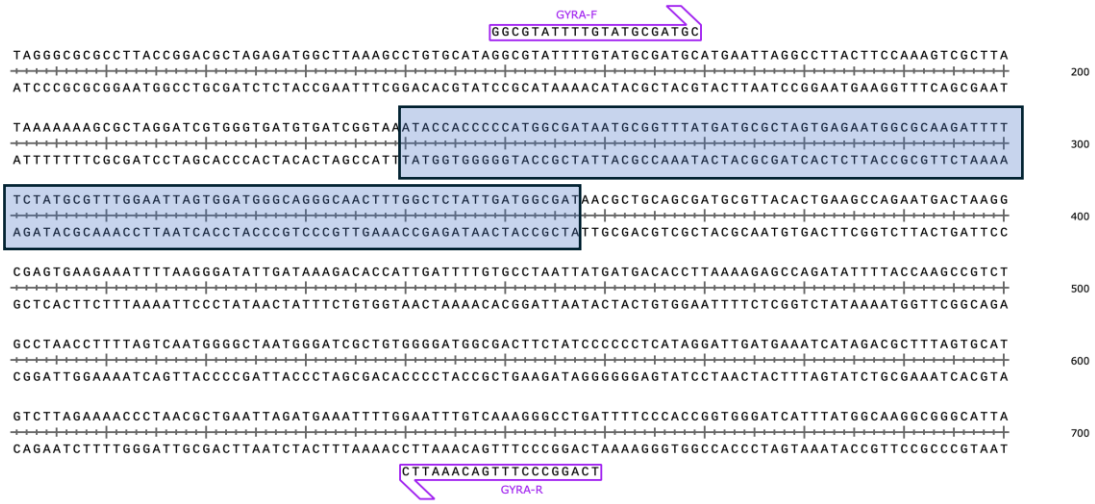


Figure 21 *- The H. pylori gyrA gene sequence from laboratory strain 11637.*
The section highlighted in blue indicates the quinolone resistance determining region (bases 240 to 357). GyrA-F and GyrA-R primer binding locations are shown by the purple arrows and mark the start and end of the 510bp target amplicon.

4.2.5 Polymerase Enzyme Tests

Sequencing was performed with the aim of identifying SNPs that can confer antimicrobial resistance. Therefore, it was important to ensure that any polymorphisms detected were actually present in the isolate, as opposed to a result of an error during the PCR process. Therefore, two different polymerase enzymes were tested, and the PCR products were sequenced to compare which would be more suitable for our purposes.

The two PCR kits that were tested were the Phusion High-Fidelity PCR kit (New England Biolabs, product code E0553L), and the DreamTaq Green PCR Master (2x) (ThermoScientific, product code K1082). Both sets of polymerases were tested with *H. pylori*

laboratory strains NCTC 11637 as well as clinical isolates from the Nottingham Strain Collection (**Table 11**). Additionally, *H. pylori* strain 26695 (ATCC 700392) [330] was tested with DreamTaq polymerase to ensure that the selected primers annealed to a range of *H. pylori* DNA templates. Laboratory strain NCTC 11637 was selected for control purposes and for mapping the sequenced clinical strains against, as it did not contain any of the levofloxacin resistance mutations of interest. Thus, changes in these locations in the clinical strains could easily be highlighted. Both sets of PCR products were sent for Sanger sequencing at Source BioScience, detailed further in section 4.2.9, page 175.

Table 11 *- H. pylori isolates selected for PCR amplification comparing DreamTaq and High-Fidelity polymerases.*

Polymerase	**DreamTaq**	**High-Fidelity**
Isolate	11637	11637
	201A	308A
	201B	308B
	256B	444A
	26695	444B

It was determined that primers GyrA-F and GyrA-R had different primer annealing temperatures from each other, so it was necessary to determine what the optimal thermocycler conditions would be. Therefore, a heat gradient test was performed in which a range of primer annealing temperatures was used. When consulting the settings used in the original publications, GyrA-F from Zhong *et al.,* (2021) [331] used an annealing temperature of

55°C, while Wang *et al.,* (2010) [199] used a temperature of 53°C for GyrA-R. Data from SnapGene® viewer, NetPrimer, and the publications from which the primer sequences were taken were all used to determine possible primer melting temperatures that should be used for the PCR process. Two different DNA polymerase enzymes were also tested to identify which would be more useful for identifying single nucleotide polymorphisms with the chosen thermocycler settings. A PCR temperature gradient of 52-57°C was used with both DreamTaq Polymerase and Phusion High-Fidelity Polymerase on laboratory strain 11637. A further temperature range of 60-68.1°C was also tested with the High-Fidelity polymerase.

Table 12 *- The PCR temperature gradient settings used with DreamTaq Polymerase and Phusion High-Fidelity Polymerase to identify the most suitable annealing temperature for the pair of primers GyrA-F and GyrA-R.*

DreamTaq, with NCTC 11637 strain	**DreamTaq, with 26695 strain**	**High-Fidelity, with NCTC 11637 strain**	**High-Fidelity, with NCTC 11637 strain**
52°C	52°C	52°C	60°C
52.9°C	52.9°C	52.9°C	61.3°C
53.4°C	53.4°C	53.4°C	62.2°C
54°C	54°C	54°C	63.2°C
54.7°C	54.7°C	54.7°C	64.7°C
56.2°C	56.2°C	56.2°C	65.7°C
57°C	57°C	57°C	66.6°C
			67.3°C
			68.1°C

For the DreamTaq polymerase, PCR tests were performed using H. pylori laboratory strain NCTC 11637 at temperature gradients of 52-57°C and then again using laboratory strain 26695 to ensure primers annealed to a variety of H. pylori DNA templates at that temperature range. High-

Fidelity polymerase PCR amplification was tested with NCTC 11637 at two temperature ranges: 52-57°C and 60-68.1°C.

The PCR products were run on an agarose gel with a 1kb ladder to check if a clear band of the expected size (510bp) was present. A temperature gradient PCR using DreamTaq polymerase enzyme was also performed using laboratory strain *H. pylori* 26695 to ensure annealing of the primers worked on other *H. pylori* DNA templates as well as NCTC 11637 (**Table 12**).

4.2.6 PCR Settings

PCR products were stored at -20°C until needed, and the PCR reactions were performed using a PCRExpress Thermocycler (Hybaid). The GyrA-F and GyrA-R primers were made up to a working stock concentration of 25μMol, and 0.5μl of each was used in every reaction along with 1μl of template DNA. Other reagents were added as per the manufacturer's instructions, and then nuclease-free water was added to bring each reaction up to a 25μl volume. When using the DreamTaq enzyme, the thermocycler conditions were set to the following conditions: 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute. The final extension step was 72°C for 5 minutes, then held at 10°C as shown in **Table 13**.

Table 13 *- Thermocycler settings for PCR protocol using DreamTaq polymerase.*

Phase	Time	Temperature	Cycles
Initial Denaturation	5 Minutes	95°C	1
Denaturation	30 Seconds	95°C	35
Annealing	30 Seconds	53°C	
Extension	1 Minute	72°C	
Final Extension	5 Minutes	72°C	1

For PCR reactions using the High-Fidelity Polymerase, the thermocycler conditions used were: a pre-denaturation step of 98°C for 30 seconds, followed by 35 cycles of 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The final extension step was at 72°C for 5 minutes, then held at 4°C (**Table 14**).

Table 14 *- Thermocycler settings for PCR protocol using High-Fidelity polymerase.*

Phase	Time	Temperature	Cycles
Initial Denaturation	30 Seconds	98°C	1
Denaturation	10 Seconds	98°C	35
Annealing	30 Seconds	65°C	
Extension	30 Seconds	72°C	
Final Extension	5 Minutes	72°C	1

4.2.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for visualising PCR products and checking the approximate amplicon size. Gels were made using 2% molecular grade agarose (Bioline), and bands visualised using GelRed Nucleic Acid Gel Stain (Biotium). For loading the High-Fidelity PCR samples into the gel, 1µl Gel Loading Dye Blue 6x (NEB, B70215) was combined with 5µl of PCR product and pipetted into each well. DreamTaq PCR products already had gel loading dye included in the Master Mix. A 1kb DNA Ladder (NEB) was used to measure the amplicon size, and the gel was run at 80 volts for 30 minutes.

4.2.8 PCR Product Purification

The *gyrA* PCR products were purified prior to sequencing to remove any components, e.g. ethanol, that may interfere with the process. For PCR products prepared using the DreamTaq polymerase, a GenElute™ PCR Clean-Up Kit (Sigma, NA1020-1KT) was used to purify 25µl of the PCR product. The manufacturer's instructions were followed, with an additional step included after the removal of ethanol from the samples. The lids of the samples were opened close to a Bunsen burner for 1 minute to allow residual ethanol to evaporate. The DNA product on the spin column was then eluted into 40µl of Qiagen Buffer EB. The elution buffer volume was

reduced from the suggested 50µl to increase DNA concentration in the eluate.

For PCR samples containing High-Fidelity polymerase, an additional gel extraction step was taken in an attempt to remove the secondary bands present in samples. Gel extraction was performed using the QIAquick gel extraction kit (catalogue number 28704). A 1% agarose gel was made, and 25µl of PCR product was loaded into each well. The gel was then run for 50 minutes at 80V, along with a 1kb ladder (NEB) to allow for identification of the correct band. The band from each well that lined up approximately with the 500bp marker from the 1kb ladder was selected. The bands in both wells for each sample were then excised with a scalpel over a GelVue UV light box (SYNGENE) and put in the same 2mL Eppendorf tube. Care was taken to remove as much excess gel as possible, while still retaining any parts of the gel containing the desired PCR product. Any secondary bands within the PCR sample were removed at this stage. A 2ml Eppendorf tube for each reaction was weighed while empty, and then weighed again with the gel fragments inside so that 3x volume of Buffer QG per 1x gel weight could be added. The clean-up then continued as described in the instructions for the kit. After product purification, 1µl of each sample was checked using gel electrophoresis on a 2% agarose gel at 80V for 40 minutes, and images were taken using UVIDoc Gel Documentation System (UVITEC). The DNA concentration in each

sample was then quantified after cleanup using a Nanodrop Spectrophotometer. If the DNA concentration reading was lower than 10ng/µl, the PCR was repeated for that sample.

4.2.9 Sanger Sequencing

Cleaned PCR samples were prepared as per the requirements of Source BioScience (UK) for Sanger sequencing. PCR samples were diluted in nuclease-free water to a concentration of 10ng/µl, and GyrA-F and GyrA-R primers were diluted to 3.2pmol/µl. For each sample, 5µl each of PCR product, forward primer and reverse primer were prepared and sent. PCR was performed in triplicate for each strain, with two of the three samples sent for sequencing and the third stored at -20°C. Upon return of the sequences, if the read quality was poor (>35), the third repeat was thawed and sequenced.

4.2.10 Sequence Analysis

Once the sequences had been received, the .seq sequence files and the .ab1 chromatogram files were opened in the Unipro UGENE program (Version 45.1) [332]. The forward and reverse sequences for the 2 repeats of each isolate (4 reads in total) were all mapped to the NCTC 11637 strain *gyrA* gene, with Mapping Minimum Similarity set to 90% and trimming set to a quality threshold of 40.

The consensus sequences of the 4 reads were checked for any discrepancies which were highlighted within the Unipro UGENE program. The consensus sequence for each isolate was then converted into the amino acid sequence, which were all aligned to the NCTC 11637 *gyrA* amino acid sequence, again using Unipro UGENE.

4.2.11 Comparing Phenotype to Genotype

The amino acid alignment file was opened in SnapGene® Viewer (Version 6.1.2), and any amino acids that differed from the NCTC 11637 sequence were highlighted. Levofloxacin MICs from the corresponding isolate were compared to any non-synonymous mutations.

4.2.12 Modelling Proteins

The sequenced 510bp *gyrA* DNA amplicons from three clinical isolates (221A, 875A, and 308A) were copied and inserted into the corresponding section of a full *gyrA* DNA gene sequence from *H. pylori* laboratory strain NCTC 11637. This was done to allow visualisation of the whole GyrA protein, instead of just the section of it that was sequenced. The DNA sequence was converted into an amino acid sequence and entered into the Phyre2 web portal for protein modelling, prediction and analysis [333], in order to

visualise the 3d structure. The Phyre2 output file (final.casp.pdb) was opened in ChimeraX-1.5 for visualisation and editing [334].

4.3 Results

4.3.1 *H. pylori* Isolate Levofloxacin Resistance

All of the *H. pylori* Isolates in the Nottingham Strain Collection had previously been tested for levofloxacin resistance (Chapter 3) [328], and E-tests were performed to provide MIC data on isolates selected for sequencing analysis. As per the EUCAST guidelines, isolates with an MIC of >1µg/ml were classified as resistant, whereas isolates with an MIC of ≤1 were classified as susceptible [329]. However, several isolates that were on the borderline of a resistant MIC (0.25-≤1) were also selected for inclusion in order to have a broader selection of MICs. These were categorised as 'borderline' for this study (**Table 15**). 38 clinical isolates were selected from the Nottingham Strain Collection, with laboratory strain 11637 also included as a control. One strain that had been initially included was later removed after it was identified as a contaminant, leaving a total of 37 clinical isolates and the 11637 control that were sequenced. The MIC range of the isolates was 0.064 to 32µg/ml.

Table 15 *- Resistance levels of H. pylori isolates from the Nottingham Strain Collection selected for culturing and sequencing.*

Isolate	MIC (µg/mL)
093A	0.25
093B	24
182A	0.125
194A	0.38
201A	0.25
201B	1
206A	0.064
206B	0.38
215A	0.064
215B	0.064
221A	0.032
221B	0.125
255A	0.25
255B	0.25
256B	0.094
274B	0.25
286A	32
308A	32
308B	32

Isolate	MIC (µg/mL)
322A	0.5
322B	0.75
335A	0.19
335B	0.19
357A	0.19
357B	0.125
402A	0.75
402B	0.5
444A	0.25
444B	0.5
642A	32
642B	32
733A	0.094
733B	0.064
875A	32
875B	32
934A	0.75
935B	32
11637	0.38

A range of MICs were selected, with 9 classified as resistant (MIC>1) (highlighted in red), 15 categorised as borderline resistant (MIC of 0.25-≤1) (highlighted in yellow), and 14 classified as susceptible (MIC <0.25) (highlighted in green). The isolate number represents the patient that the H. pylori sample was cultured from. The 'A' and 'B' in the isolate name indicate the area of the stomach that the biopsy was taken from within that patient (A = antrum, B = corpus). H. pylori laboratory strain NCTC 11637 was included as a control.

4.3.2 Strain Growth and Genomic DNA Extraction

The extracted genomic DNA was run on an agarose gel to ensure that sufficient amounts of high molecular weight genetic material

were available for downstream use. Growth of several of the *H. pylori* cultures was initially poor, and the DNA yield was low. For these strains, further culturing was undertaken to increase yields.

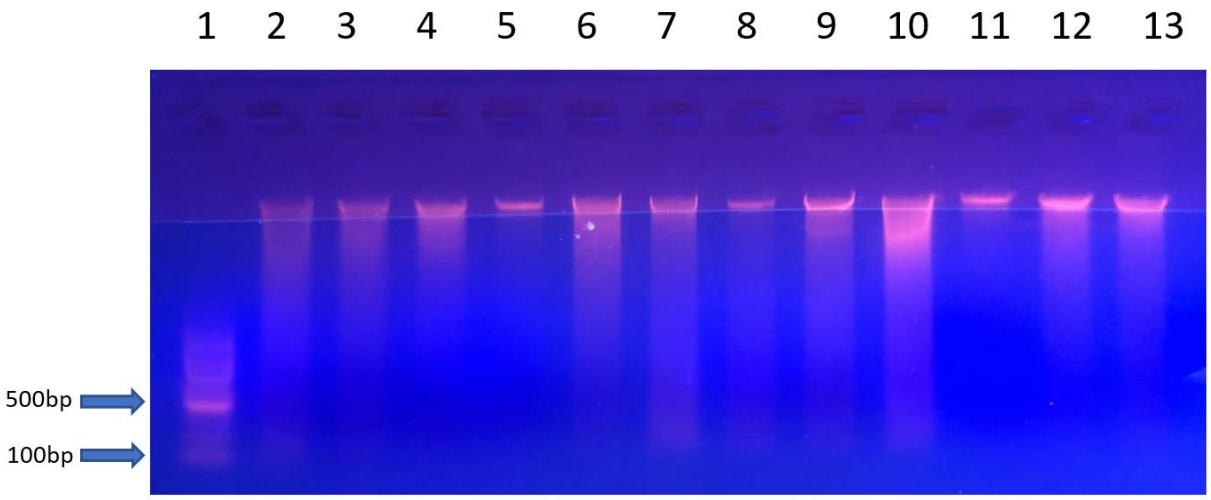


***Figure 22** - Agarose gel separation of gDNA extracted from H. pylori isolates.*

(Left to right) Lane 1 contains 5µl of 100bp DNA ladder (NEB). Lanes 2-13 contain high molecular weight gDNA extracted from H. pylori isolates 642B, 875B, 286A, 613A, 733B, 93A, 93B, 444A, 201A, 201B, 934A and 733B (repeat) respectively.

Isolate 613A **(Figure 22 , Lane 5)** which had been initially selected for sequencing was found to be very difficult to culture and grow in enough quantity. Further investigation led to the conclusion that stocks of this strain were contaminated, so it was removed from the study.

4.3.3 Heat Gradient PCR and DNA Polymerase Enzyme Optimization

The objective of this study was to identify resistance conferring SNPs in the *gyrA* gene of *H. pylori* via Sanger sequencing of the PCR products. Therefore, it was necessary to optimise the PCR protocol to ensure that DNA sequence errors were not introduced at that stage, which could obfuscate the identification of legitimate resistance mutations. The heat gradient tests were initially performed comparing two different DNA polymerases on genomic DNA template from *H. pylori* laboratory strains. DreamTaq and High-Fidelity polymerases were tested on PCRs from strain 11637 DNA, with a range of annealing temperatures from 52-57°C. The gel of the DreamTaq PCR products showed bands of the predicted amplicon size all of a similar yield and quality (**Figure 23 A**). The initial 52-57°C range for the High-Fidelity polymerase with 11637 showed poor results, however, with additional bands and smears. Because of this, the same High-Fidelity enzyme PCR test was repeated but with an annealing temperature range of 60-68.1°C, however, the gel of the higher annealing temperature PCR products was again poor compared to the DreamTaq products (**Figure 23 B**).

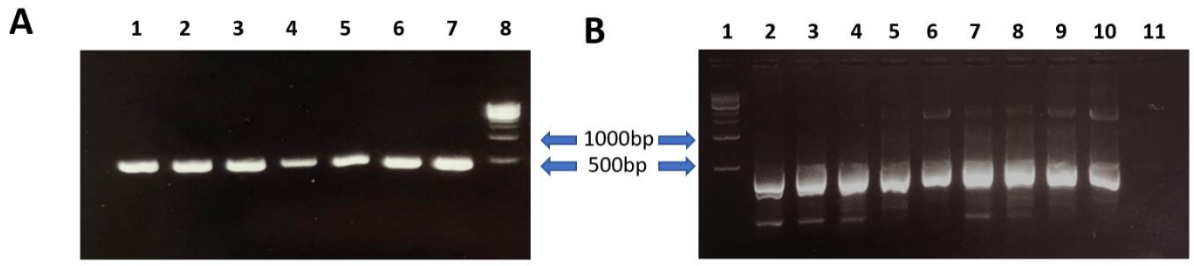


***Figure 23**- Agarose gel separation of gyrA gene PCR products from H. pylori lab strains and obtained using a temperature gradient for the annealing step of the PCR process.*

A) (Left to Right) PCR products obtained using DreamTaq polymerase (ThermoScientific). Lanes 1-7 contain PCR products of ~500bp in size obtained from lab strain 11637 with a gradient of annealing temperatures as follows; 52°C, 52.9°C, 53.4°C, 54°C, 54.7°C, 56.2°C, 57°C respectively. Lane 8 contains 5µl of 1kbp DNA ladder (NEB) B) PCR products obtained using Phusion high-fidelity PCR kit (NEB). (Left to right) Lane 1 contains 5µl of 1kbp DNA ladder (NEB). Lanes 2-12 contain PCR products of varying size obtained from lab strain 11637 with a gradient of annealing temperatures as follows; 60°C, 61.3°C, 62.2°C, 63.2°C, 64.7°C, 65.7°C, 66.6°C, 67.3°C, and 68.1°C. Lane 11 contains a no template PCR negative control.

The High-Fidelity polymerase was also tested with the same temperature gradient (52-57°C) but with a different *H. pylori* laboratory strain, 26695 (**Figure 24**). This was to ensure that the primers worked efficiently with a range of *H. pylori* strains, and to further discern if any PCR annealing temperature was more effective. The gel bands for DreamTaq were mostly consistent, and did not seem to indicate that PCR performance was significantly better for any of the annealing temperatures within the range. Therefore, a temperature of 53°C was selected for DreamTaq polymerase PCR as this was the annealing temperature previously used by Wang *et al*., [199] who used the same reverse primer that was being used for this study.

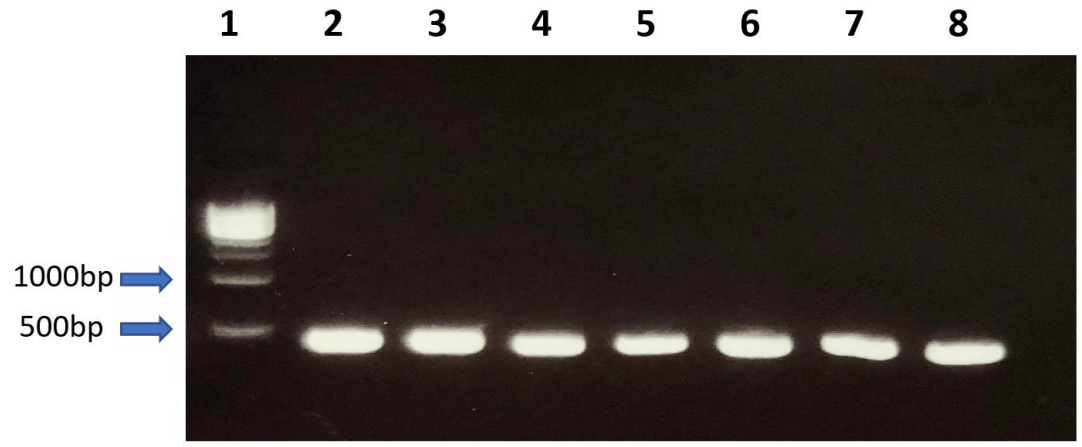


Figure 24 *- Agarose gel separation of gyrA gene PCR product from H. pylori laboratory strain 26695 and obtained using a temperature gradient for the annealing step of the PCR process. PCR products obtained using DreamTaq polymerase (ThermoScientific). Lane 1 contains 5µl of 1kbp DNA ladder (NEB) Lanes 2-8 contain PCR products of ~500bp in size obtained from lab strain 26695 with a gradient of annealing temperatures as follows; 52°C, 52.9°C, 53.4°C, 54°C, 54.7°C, 56.2°C, 57°C respectively.*

4.3.4 Troubleshooting

Due to the presence of secondary bands and smears appearing in the gels for the High-Fidelity polymerase samples, additional testing was done to troubleshoot the PCR protocol. A higher range of annealing temperatures was tested to see if non-specific DNA annealing was the cause of the additional bands. Steps were carefully taken when testing the High-Fidelity polymerase to remove any other sources of error that might be causing double banding, such as premature primer or template degradation. The PCR reagents were all combined on ice, and the lid of the thermocycler was preheated to 98°C. All other reagents and the

template DNA were added to the PCR tubes, with the High-Fidelity polymerase added last, and the samples quickly moved to the thermocycler to avoid non-specific transcription from occurring. However, gel images continued to show the presence of additional bands and smears.

Therefore, gel excision clean-up of the PCR products was also attempted. While this did remove the worst of the smearing and some of the low molecular weight bands (< 500bp), there still appeared to be faint bands present at ~1000bp (**Figure 25**), and the PCR product yield was also reduced. These samples, along with the PCR amplicons obtained using DreamTaq polymerase, were sent for Sanger sequencing to assess the quality of the DNA sequences produced and compare their usefulness in identifying possible SNPs.

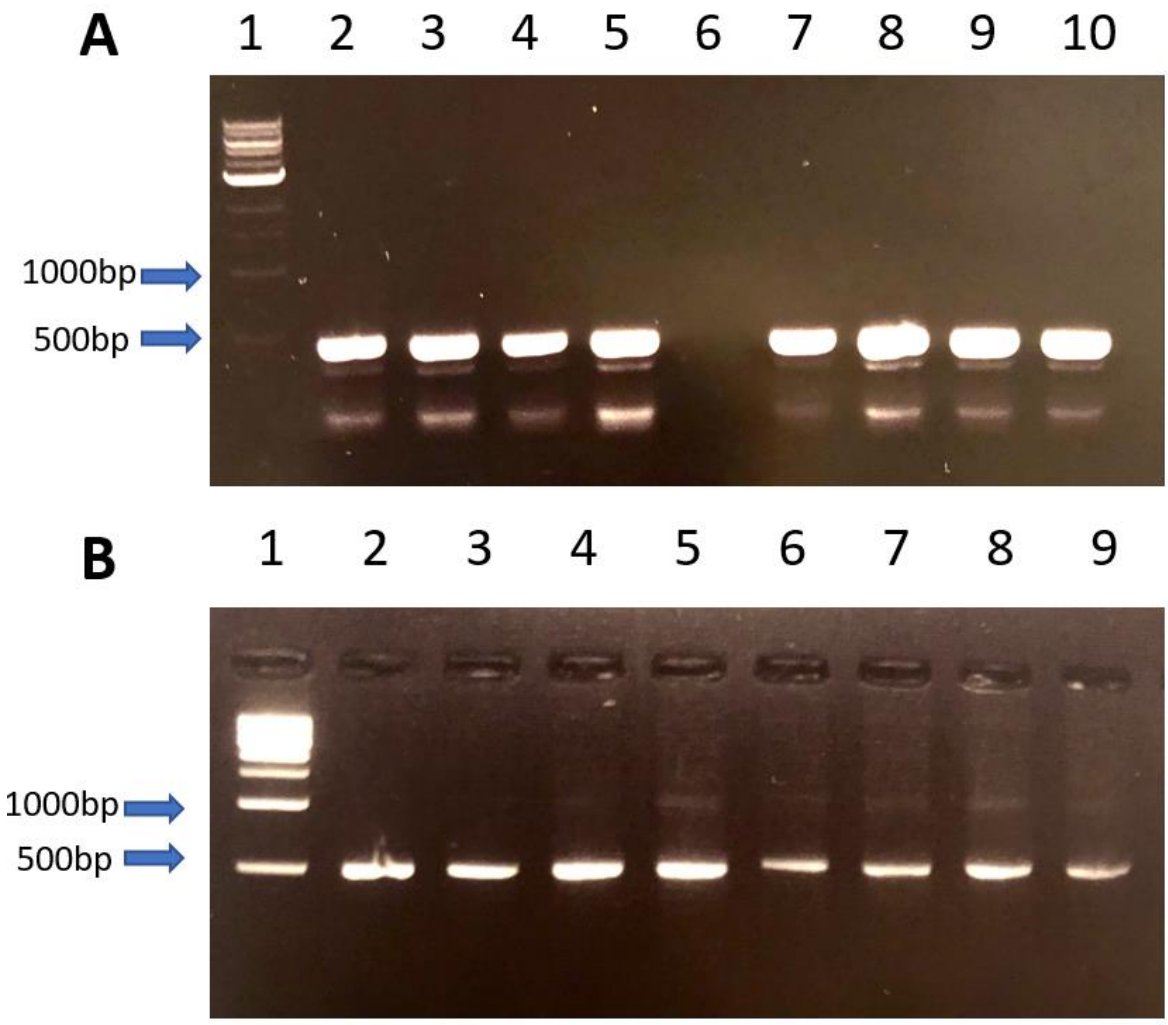


Figure 25 *- Agarose gel separation of PCR products obtained using the Phusion High-Fidelity PCR kit (NEB) from H. pylori isolates - before and after clean-up.*

A) (Left to right) Lane 1 contains 5µl of 1kbp DNA ladder (NEB). Lanes 2-10 (excluding lane 6) contain PCR products from the following H. pylori isolates; lanes 2, 3, 4 and 5 contain isolate 93A, lane 6 is blank, lanes 7, 8, 9 and 10 contain isolate 93B. A band at the size of the expected PCR fragment of ~500bp is visible, and additional secondary PCR products are also present. B) (Left to right) Lane 1 contains 5µl of 1kbp DNA ladder (NEB). Lanes 2-9 contain PCR products from the following H. pylori isolates that have been purified using the QIAquick gel extraction kit; lanes 2 and 3 contain isolates 93A, lanes 4 and 5 contain isolate 93B, lanes 6 and 7 contain isolate 642A, and lanes 8 and 9 contain isolate 642B.

4.3.5 PCR and Sanger Sequencing of Clinical Isolates

The *gyrA* genes of the clinical isolates were amplified using DreamTaq polymerase enzyme and primers GyrA-F and GyrA-R (described in **Section 4.2**) and then products were purified using the QIAquick gel extraction kit (**Figure 26**) before being sent to Source BioScience for Sanger sequencing. Sequencing data was returned with information of N-base calls (percentage of bases that could not be identified during base calling), length of sequencing read, and Quality Value (a metric of confidence of the assigned base for each signal during sequencing).

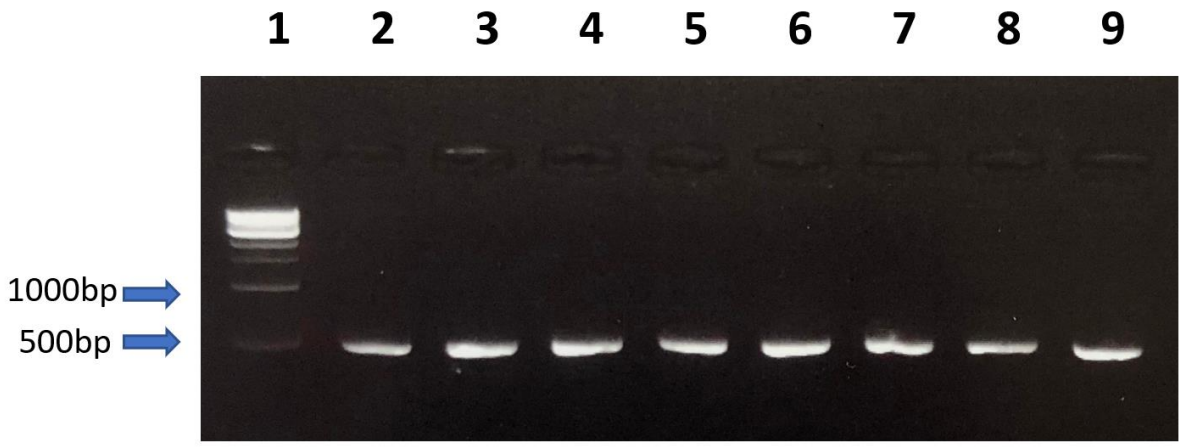


Figure 26 *- Agarose gel separation of PCR products obtained using DreamTaq polymerase enzyme (ThermoScientific) from H. pylori clinical isolates.*

(Left to right) Lane 1 contains 5µl of 1kbp DNA ladder (NEB). Lanes 2-9 contain cleaned-up PCR products of ~500bp in size from the following H. pylori isolates; lanes 2 and 3 - 93A, lanes 4 and 5 - 93B, lanes 6 and 7 - 642A, lanes 8 and 9 - 642B.

The categorisations provided by Source Bioscience indicated that a Quality Value of >35 was 'Good', 20-35 was 'Intermediate' and <20 was 'Poor'. The DreamTaq PCR products all returned with Quality Values consistently over 54, with the exception of isolate 875B (Quality Value of 13). Sequencing of *gyrA* from this strain was repeated. The N % value was between 1-4% before the ends of the reads were trimmed, and all the read lengths were ~480bp long. However, the Quality Value for the High-Fidelity reads were all <20 and categorised as 'Poor'. The percentage of N base calls in the High-Fidelity PCR product set was comparatively high, with several of the reads having more than 20% of the bases classed as unidentifiable. The read length also varied widely, with some sequences being less than half the expected 510bp of the amplicon, and isolate 308A only having 91bp (**Table 16**)

Table 16 *- Comparison of N%, Length of Read and Quality Value for a sample of DreamTaq and High-Fidelity PCR products after Sanger sequencing.*

Isolate and Primer	DreamTaq Polymerase			High-Fidelity Polymerase		
	N (%)	Length of Read	Quality Value	N (%)	Length of Read	Quality Value
308A GyrAF	4	479	56	17	446	15
308A GyrAR	1	480	56	8	91	18
308B GyrAF	3	480	57	10	287	18
308B GyrAR	2	478	57	18	363	14
444A GyrAF	2	478	56	13	203	17
444A GyrAR	2	479	57	24	432	12
444B GyrAF	3	483	54	19	238	13
444B GyrAR	3	478	56	17	313	17
Mean	2.5	479.38	56.13	15.75	296.63	15.5
Range	1-4	478-480	54-57	8-24	91-446	12-18

The data for 4 isolates are shown here, with sequencing information of both the forward (GyrAF) and reverse (GyrAR) PCR products. The Sanger sequencing and quality report were provided by Source Bioscience. Quality Values >35 are classified as 'Good', values 20-35 are classified as 'Intermediate' and values <20 are 'Poor'.

4.3.6 Chromatograms

The chromatogram and sequence files (.ab1 and .seq, respectively) for the forward and reverse primer reads were opened using Unipro UGENE (Version 45.1) and compared. The High-Fidelity polymerase PCR products returned a chromatogram with unclear double signals in many places, with a large proportion of N bases and a significantly lower base-call certainty than the DNA amplicons acquired using DreamTaq polymerase (**Figure 27**). Additional bases were inserted into the consensus sequence due to overlapping signals, which meant the sequences were misaligned to other isolates and difficult to compare. These factors made the High-Fidelity polymerase unsuitable for detecting SNPs, so the remainder of the isolates were amplified with DreamTaq polymerase and sent for Sanger sequencing.

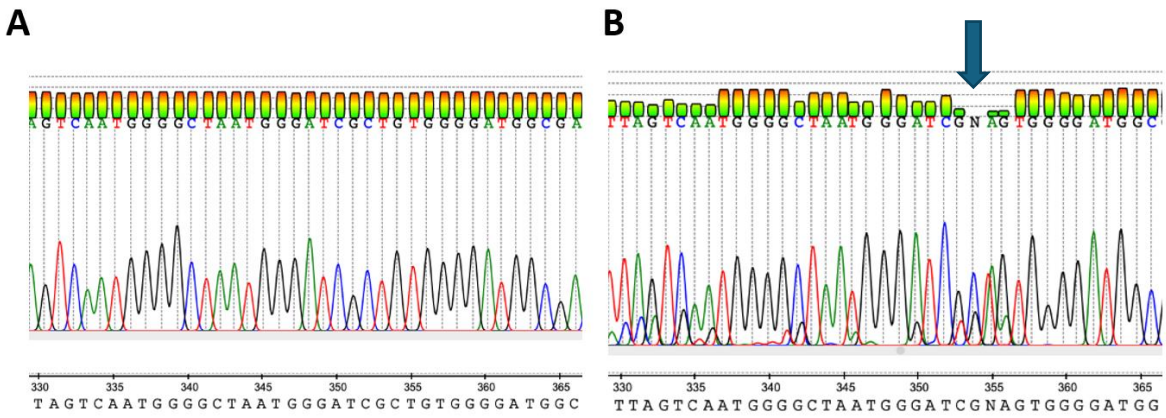


Figure 27 *- An example section of the chromatogram and DNA Sanger sequencing data from H. pylori laboratory isolate 11637.*

Obtained using A) DreamTaq polymerase PCR and B) High-Fidelity polymerase. The base calling for the High-Fidelity had a lower certainty and contained overlapping signals for some bases. The blue arrow points to an example of an 'N' base call, where a nucleotide could not be identified. There are also additional bases inserted into the sequence. The red/yellow/green bars above each base represent confidence that the base has been correctly called. Chromatograms viewed using Unipro UGENE [332].

Each isolate was PCR-amplified two times independently so that both repeats could be sent for Sanger sequencing and compared to each other for accuracy. The forward and reverse amplicons of each repeat were sequenced, meaning that each *H. pylori* isolate had four DNA sequence files. The four Sanger sequencing files for each isolate were imported into Unipro UGENE, and the ends of each read were trimmed to remove sections of the sequences with lower base-call certainty. These four reads were then aligned to the published NCTC 11637 *gyrA* sequence (National Center for Biotechnology Information (NCBI) Accession number LS483488), and SNPs were highlighted (**Figure 28**)

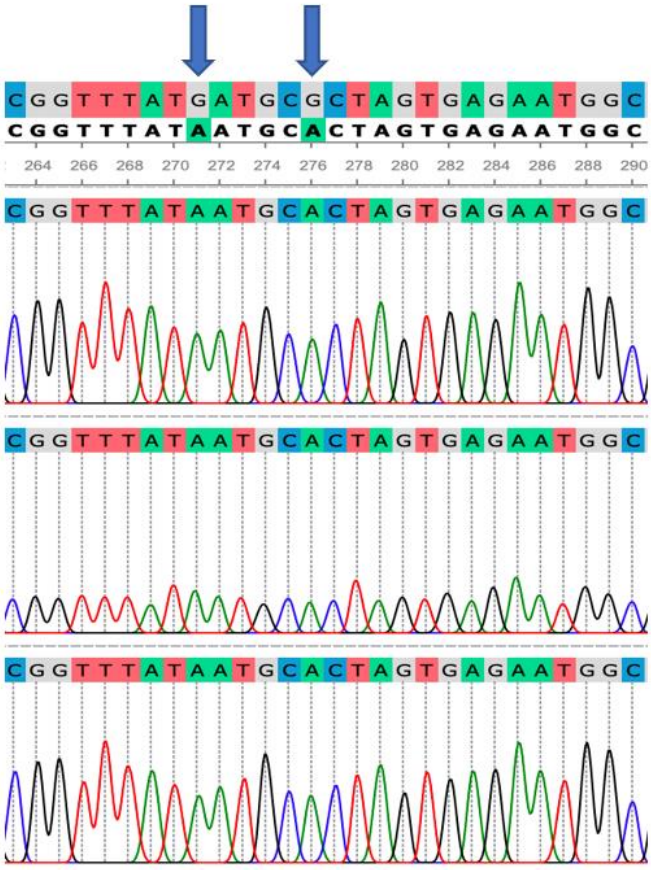


Figure 28 *- A Chromatogram showing a consensus of 3 reads of isolate 308B, mapped to the reference strain 11637.*

The mutation G271A corresponds to a substitution of amino acid 91 from aspartic acid to asparagine, a known resistance gene. The G276A mutation is silent, as the triplets GCA and GCG both encode the same amino acid (alanine).

After converting the nucleotide sequence into a protein sequence using Unipro UGENE, the amino acid substitutions for each isolate displayed in **Figure 29** and **Table 15** were consistent across both PCR repeats with no discrepancies. From there, the consensus sequence was extracted and converted into the corresponding amino acid sequence, allowing for the identification of any non-synonymous mutations.

4.3.7 Amino Acid Substitutions

Several previously unreported mutations were noted. A single isolate, 182A, contained an amino acid substitution at position 89 from a valine (GTT) to an isoleucine (ATT). The MIC of isolate 182A was 0.125 and classed as susceptible. Isolates 308A and 308B both contained a novel mutation that led to amino acid substitution Phe149Leu. Both of these isolates were highly resistant to levofloxacin (MIC >32), however they also contained the known resistance gene Asp91Asn. Three isolates had a substitution at amino acid site 63, with susceptible isolates 221A and 221B (MIC 0.032 and 0.125 respectively) having serine (TCC) replaced by phenylalanine (TTC). The third substitution was at site 63, with the serine residue (TCC) replaced by proline (CCC), which was only found in one isolate, 255B. This isolate had a borderline resistant MIC of 0.25. However, the corresponding antral isolate (255A) from the same patient did not contain this substitution, instead having the serine (TCC) amino acid, the same as in the 11637 control strain.

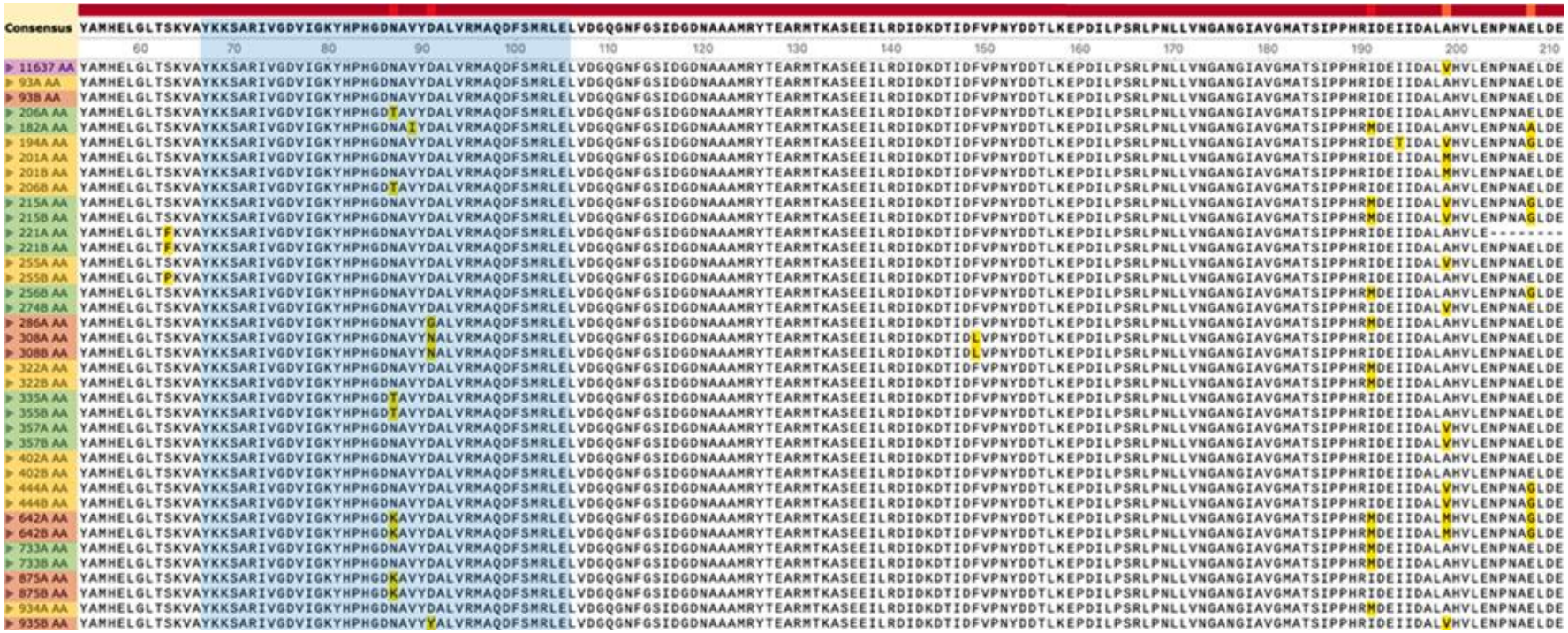


Figure 29 *- Amino acid consensus sequences of the gyrA gene of H. pylori clinical isolates, aligned to the amino acid sequence of laboratory strain 11637.*

The light blue highlighted box indicates the location of the Quinolone Resistance Determining Region. Isolate names on the left of the image are highlighted to denote levofloxacin resistance classification (red for resistant, orange for borderline resistant and green for susceptible, with laboratory strain 11637 highlighted in purple). Amino acid residues that are different to those in the 11637 sequence are highlighted in yellow. Sequences aligned using SnapGene® Viewer (Version 6.1.2).

H. pylori isolate 194A was the only sequence with a substitution at amino acid position 194, with isoleucine (ATC) replaced by threonine (ACC). This isolate had a borderline resistant MIC of 0.38, and did not contain any mutations in the QRDR. At amino acid position 199, laboratory strain 11637 contains a valine residue (**Figure 29**). However, the consensus of all the amino acid sequences indicates that alanine is more common at that locus. Another four isolates had a methionine (Met) residue instead of valine at position 199. The valine and alanine polymorphisms did not seem to have any connection to MIC based on this data, however the Val199Met change was only present in isolates that had resistant or borderline resistant MICs (**Table 17**). Isolates 642A and 642B both had an MIC of 32, but also contained the known resistance substitution Asn87Lys, whereas isolates 201A and 201B (MICs of 0.25 and 1 respectively) did not have any other identifiable mutations.

Amino acid substitutions were also identified in some isolates at positions 51, 52 and 208, however these fell outside the expected amplicon range. As amino acid 208 comes after the binding site of the reverse primer on the gene sequence it was only picked up by the very end of the forward primer amplicon. The inverse is true of amino acids 51 and 52, and as such those substitutions were not included within the results.

Table 17 *- gyrA amino acid substitutions identified in H. pylori clinical isolates, compared to levofloxacin MIC (µg/mL).*

Isolate	MIC (µg/mL)	*gyrA* Mutations
286A	32	Asp91Gly + Ile191Met
308A	32	Asp91Gly + Phe149Leu
308B	32	Asp91Gly + Phe149Leu
642A	32	Asn87Lys + Ile191Met + Val199Met
642B	32	Asn87Lys + Ile191Met + Val199Met
875A	32	Asn87Lys
875B	32	Asn87Lys
935B	32	Asp91Tyr
093B	24	-
201B	1	Val199Met
322B	0.75	Ile191Met
402A	0.75	-
934A	0.75	Ile191Met
322A	0.5	Ile191Met
402B	0.5	-
444B	0.5	Glu208Gly
194A	0.38	Ile194Thr
206B	0.38	Asn87Thr

Isolate	MIC (µg/mL)	*gyrA* Mutations
093A	0.25	-
201A	0.25	Val199Met
255A	0.25	-
255B	0.25	Ser63Pro
274B	0.25	-
444A	0.25	-
335A	0.19	Asn87Thr
335B	0.19	Asn87Thr
357A	0.19	-
182A	0.125	Val89Ile + Ile191Met
221B	0.125	Ser63Phe
357B	0.125	-
256B	0.094	Ile191Met
733A	0.094	Ile191Met
206A	0.064	Asn87Thr
215A	0.064	Ile191Met
215B	0.064	Ile191Met
733B	0.064	Ile191Met
221A	0.032	Ser63Phe

MIC ranges are highlighted to indicate classification (red=resistant, yellow=borderline resistant, green=susceptible). Isolates with a dash symbol did not contain any substitutions in comparison to the laboratory strain 11637 amino acid sequence. Val199Met substitutions were included, however Val199Ala substitutions were not, as the consensus indicates that alanine is the more commonly found amino acid at this position.

4.3.8 Incidence of Substitutions at Amino Acid positions 87 and 91

Of the 9 isolates classed as resistant, 8 of them (88%) had a substitution at amino acid position 87 or 91. The substitution that was the most common in the resistant isolates was Asn87Lys, which was detected in 4 of the 9 (44.4%) resistant isolates. The

mutation leading to the Asn87Thr substitution on the other hand was the only QRDR mutation that was present in any of the susceptible isolates (4/28, 14.3%) and was not present in any of the resistant isolates. No isolate had both amino acid 87 and 91 substitutions together. Only one resistant isolate did not contain a substitution at either position 87 or 91, which was isolate 93B (MIC 24), and there were no susceptible isolates with a substitution at position 91 (**Table 18**)

Table 18 *- Amino acid substitutes identified at position 87 and 91 in the gyrA gene of clinical H. pylori isolates.*

Mutations	Resistant (%)	Susceptible (%)	Total (%)
87	4/9 (44.4)	4/28 (14.3)	8/37 (21.6)
91	4/9 (44.4)	0/28 (0)	4/37 (10.8)
Either 87 or 91	8/9 (88.9)	4/28 (14.3)	12/37 (32.4)
Neither 87 or 91	1/9 (4.0)	24/28 (96.0)	25/37 (67.6)
Asn87Thr	0/9 (0)	4/28 (14.3)	4/37 (10.8)
Asn87Lys	4/9 (44.4)	0/28 (0)	4/37 (10.8)
Asp91Gly	1/9 (11.1)	0/28 (0)	1/37 (2.7)
Asp91Asn	2/9 (22.2)	0/28 (0)	2/37 (5.4)
Asp91Tyr	1/9 (11.1)	0/28 (0)	1/37 (2.7)

Amino acid substitutions at positions 87 and 91 have been previously identified as conferring levofloxacin resistance to H. pylori [292]. The data are grouped by amino acid position and then broken down into specific amino acid substitutions.

4.3.9 Protein Modelling and Visualisation

Three *gyrA* sequences from the clinical isolates were selected for protein modelling using Phyre2 [333] and visualised using ChimeraX [334]. Isolates 875A and 308A were chosen because they had amino acid changes at positions 87 and 91 respectively and had a resistant phenotype. Isolate 221A was also chosen for comparison, as it had a susceptible phenotype and no detected QRDR substitutions. The highlighted amino acids in **Figure 30** showed the locations of the substitutions within the structure, but overlaying the images did not appear to reveal any changes in the secondary or tertiary structures of the QRDR region between the three isolates. However, when viewing the structure of the QRDR in

a sphere model, changes between the three become more apparent (**Figure 30**).

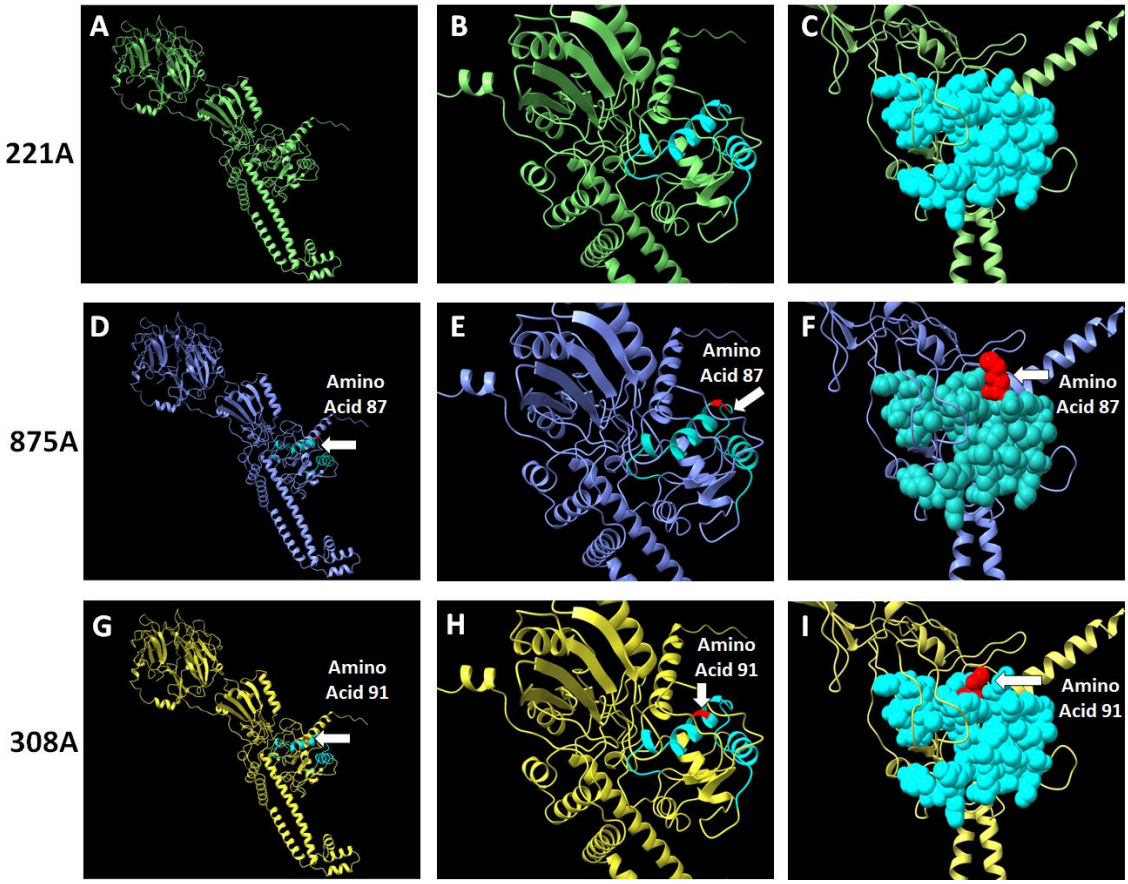


Figure 30 *- Images of protein models based on the gyrA sequences of 3 H. pylori clinical isolates, two levofloxacin-resistant and one susceptible, displaying the full protein (left column) and a close up of the Quinolone Resistance Determining Region (QRDR) (middle and right columns).*

The QRDR is highlighted in cyan, and amino acid substitutions that confer quinolone resistance are highlighted in red. Images A, B and C (green) are of isolate 221A (MIC 0.032) which contained no QRDR substitutions and was susceptible to levofloxacin. Images D, E and F (purple) are of isolate 875A (MIC 32) with an Asn87Lys substitution, and images G, H and I (yellow) are of isolate 308A (MIC 32) with a Asp91Gly substitution. The first column shows the entire gyrA protein, and the second column shows the QRDR in more detail in a ribbon model. The third column displays the QRDR in sphere model.

4.4 Discussion

4.4.1 Main Findings

In this study, it was found that PCR using DreamTaq polymerase could provide good quality amplicons and reliability for the detection of SNPs within the *gyrA* gene, in comparison to High-Fidelity polymerase. It was also found that amino acid changes at positions 87 and 91 were linked with high levofloxacin MICs, and in particular Asn87Lys, Asp91Gly, and Asp91Tyr. Asn87Thr appears to be linked to low levofloxacin MIC. It was also found that there may be other factors that can influence resistance, as one isolate displayed a highly resistant phenotype without any QRDR mutations. Several novel mutations in the *gyrA* gene were identified, but further work would need to be performed to identify their relationship with levofloxacin resistance.

4.4.2 PCR High-Fidelity and DreamTaq Enzyme Performance

When comparing polymerase enzymes for the amplification of the *gyrA* gene, DreamTaq consistently produced higher yields, with cleaner amplicons when viewed on an agarose gel than when High-Fidelity polymerase had been used. Sanger sequencing results showed that chromatograms obtained from High-Fidelity acquired

DNA sequences had low sequence certainty resulting in a number of ambiguous base calls in the sequence.

There are a range of reasons why this high background noise might have occurred. Unfortunately, due to time constraints, it was not possible to fully troubleshoot the issue. However, there are some conclusions that can be drawn about why the High-Fidelity polymerase PCR failed based on the agarose gel patterns and the chromatograms returned from Sanger sequencing. It is unlikely that the Sanger sequencing itself was problematic, as similar issues were not encountered when sequencing DreamTaq polymerase amplicons, while separate DreamTaq repeats encountered the same issue despite both sets of samples receiving the same preparation and treatment.

The chromatograms did not appear to show two distinct overlapping sequences, which means that it is unlikely that the primers were binding non-specifically in the template DNA sequence, which would have caused amplification of sequences outside the target region. Furthermore, competing sequences, where the primers bind to a sequence that repeats multiple times in the sequence are an unlikely culprit. Additionally, if the primer design was the cause of the problem, it is likely that the same issues would have also been observed in DreamTaq samples, as the same primers were used for both sets of polymerase reactions.

However, no evidence of additional non-target sequences were seen in DreamTaq samples.

Primer GyrA-F had a GC content of 47.6% with a length of 21 nucleotides, and primer GyrA-R had a GC content of 45%, and was 20 nucleotides long, both within the recommended parameters for primer design (20-30 nucleotides and 40-60% GC content) as laid out by NEB [335]. DNA template concentrations added to the reactions were correct as per the protocol and had appropriate GC content (GC of *H. pylori* is around 38% [336]). The expected amplicon length of 510bp was also not outside the limits of size for the polymerase, which can reliably amplify DNA templates of 10-20kb in length according to the manufacturer [337].

A possible cause of the issue is primer degradation due to the exonuclease activity of High-Fidelity polymerase. The NEB Phusion High-Fidelity polymerase has a 3'-5' exonuclease that acts as a 'proofreader', digesting mismatched nucleotides that have been incorporated into the extending strand before the polymerase continues. Incorrectly added nucleotides cause a slight change in the geometry of the minor groove of the DNA strand, which causes the extending primer strand to be relocated to the exonuclease site of the polymerase. There, the 3'-5' exonuclease active site of the enzyme removes the mis-incorporated base, and the strand then moves back to the polymerisation domain and the extension

process continues [338]. However, single-stranded DNA primers can also be degraded at this 3’-5’ exonuclease site [335]. Although measures were taken to ensure degradation was reduced, (i.e. assembly on ice and adding polymerase last before immediately transferring to thermocycler), further precautions could be taken such as preparing smaller batches of samples for PCR which would reduce the overall time of PCR assembly.

If protocol changes do not alleviate primer degradation, another solution would be to use primers that contain a phosphorothioate backbone, in which the oxygen atom in the phosphodiester bonds which link the DNA nucleotides together is replaced with a sulphur atom. This makes the primers resistant to endonuclease activity and decreases degradation [339]. Aside from the high-fidelity polymerase, *H. pylori* itself contains a number of endonucleases that may cause primer degradation [340]. Increasing proteinase K incubation time to 16 hours before may help alleviate primer restriction (advice from Dr. Daniel Whiley, Nottingham Trent University).

Time constraints stopped us from further elucidating why the HF PCR was not giving the expected results. Given more time, it is possible that a method could be developed that uses High-Fidelity polymerase successfully and might provide improved results. In this instance however, the results obtained from DreamTaq

polymerase were of a high enough quality to allow SNP identification with a good level of confidence, as evidenced by the four sequenced amplicons for each isolate (2x forward and 2x reverse) all being in consensus with one another.

4.4.3 Temperature Gradient

There was very little discernible difference between PCR results for the annealing temperature gradient tests. For the DreamTaq polymerase PCR samples, the initial range of temperatures all showed expected DNA amplicons of good quantity and quality, so further investigation was not required. However, for Phusion High-Fidelity PCR samples, the gradients tested produced poor results. If the adjustments to the protocol have no effect on the outcome of the products, further annealing temperature ranges could be tested in the future. Guidance from New England Biolabs suggests that annealing temperatures for Phusion polymerase is generally around 3 degrees higher than for Taq-based polymerases [335], and this should be considered.

4.4.4 GyrA Amino Acid Substitution at Locations of Interest 87 and 91

The results shown in this study found that there were eight *H. pylori* isolates with an amino acid substitution at position 87 in total, with four of those containing a threonine and four of them containing a lysine residue. No isoleucine substitutions were

identified. The presence of any substitution in general at position 87 was not enough to indicate phenotypic resistance, however, this study supports the conclusions drawn in other papers [307], [309], [310] that the presence of a lysine at position 87 is indicative of a levofloxacin resistant phenotype.

All isolates containing Asn87Lys were resistant to levofloxacin and showed high MIC values of 32µg/ml - the maximum MIC value that could be measured by E-test strips. This means that any MIC values that were higher than 32µg/ml could not be differentiated, and in the future, it may be worth considering methods of measuring higher MIC levels. The E-test strips in Cattoir *et al*., (2007) provided measurements of up to 64µg/ml [307], however these are no longer available for use.

Conversely, the four isolates that were identified as contained a threonine residue at position 87 were all classified as phenotypically susceptible to levofloxacin (MIC range 0.064-0.38). This is in keeping with findings previously published by Cattoir *et al*. (2007), which state that Thr87 strains were more susceptible to fluoroquinolones than isolates containing an Asn87 codon [307]. It may be useful to consider identifying isolates containing this mutation from patient samples in the future, as patients harbouring a highly levofloxacin-susceptible strain of *H. pylori* would likely be good candidates for a treatment therapy containing

a fluoroquinolone. However, no isolates containing both a 'susceptible' Thr87 codon and a 'resistant' position 91 codon were found, and the effect that this incongruous combination might have on phenotypic resistance might provide some insights into the mechanisms of *gyrA* resistance.

All four of the isolates that contained any kind of substitution at amino acid position 91of the *gyrA* gene were found to be highly resistant, with all of them having the highest measurable MIC of 32µg/ml. This was the case no matter what amino acid substitution was present in the place of the wild-type aspartic acid residue. All three of the Asp91 substitutions identified in this study (Gly, Asn and Tyr) were closely related to levofloxacin resistance, which is in agreement with literature that has previously been published [309], [310], [313], [341]. The alanine and histidine mutations that were identified in Wang *et al*., (2001) [311] and Garcia *et al*., (2011) [308] respectively were not seen in the course of this study.

Interestingly, isolate 093B was strongly phenotypically resistant to levofloxacin with an MIC of 24µg/mL, despite not having any amino acid substitutions at positions 87 or 91. The reasons for this isolate having a resistance phenotype are as yet unknown, although several possibilities may cause this effect, such as currently unknown DNA gyrase mutations outside of the QRDR, or the

presence of other resistance mechanisms like outer membrane proteins [342]. Resistant isolates that do not have any notable *gyrA* mutations have been described several times in the past [199], [300], [301], [302] which further indicates that an enigmatic resistance mechanism may be at play.

The presence of plasmids containing resistance genes might also provide an explanation, although in general *H. pylori* resistance genes are found chromosomally [343]. Unfortunately, no double amino acid 87 and 91 mutations were detected in this set of isolates, so it was not possible to draw any conclusions about the relationship that containing both mutations might have on resistance phenotype.

While sequencing the QRDR of a *H. pylori* isolate is not a foolproof way of predicting an isolate's phenotypic resistance to levofloxacin, it can still provide valuable information that can be used to inform treatment options. Based on the results shown in this study and in previous research, it can be concluded that the presence of a lysine residue at amino acid position 87, or of a glycine, asparagine or tyrosine residue at position 91, can be a strong indicator that the isolate will harbour a levofloxacin-resistant phenotype. If a threonine residue is detected at position 87, then the strain is likely to be levofloxacin susceptible. As such, PCR testing of the *gyrA*

region as presented in this research is likely to provide information that can accurately assess strain susceptibility to levofloxacin.

4.4.5 Amino Acid Substitutions at Other Loci

Several amino acid substitutions were found within the *gyrA* genes of the selected isolates. A substitution that has not previously been reported was found at amino acid position Val89Ile in a single isolate, 182A. This isolate has an MIC of 0.125µg/ml, and as such it does not seem likely that this mutation is linked to antibiotic resistance, although with only a single isolate containing this mutation, it cannot be confirmed currently. The first base in the codon 89 triplet (nucleotide 265) had substituted a G nucleotide with an A. Interestingly, the base before it (nucleotide 264) had also mutated from a G to an A, however, this was a synonymous mutation of GCG (Ala) to GCA (Ala). This mutation was of note because it falls directly in between the two common amino acid changes of interest (87 and 91) and would lie within proximity of the fluoroquinolone binding site.

Substitutions at amino acid position 63 were found in 3 isolates. The paired isolates 221A and 221B both contained the Lys63Phe substitution, and had an MIC of 0.125µg/ml, which suggests that this amino acid is not linked to levofloxacin resistance. The isolate 255B also had a substitution at this location, however it contained a 63Pro instead. This isolate had been measured as having a

susceptible MIC of 0.25µg/ml, however the paired isolate that came from the antrum of the same patient (255A) did not contain the same mutation. This will be discussed in more detail in the next section.

The mutation Phe149Leu was identified in the paired isolates 308A and 308B, which were both highly resistant to levofloxacin with MICs of 32µg/ml. This mutation has not been noted in previous published literature. However, these two isolates also contained the Asp91Asn mutation which is already known to be strongly associated with levofloxacin resistance, so it cannot be said with any certainty if the 149Leu mutation has any impact on phenotypic resistance without further examples.

Some of the resistance substitutions that have been previously described were absent from this dataset. Mutations at locations of interest 85, 86, and 88 have been previously found in resistant isolates from South Korea, France, and Canada respectively. Specifically, these studies reported a Gly85Cys substitution in Rhie *et al*., (2020) [301], Asp86Asn in (Cattoir *et al*., 2007) [307] and Ala88Val in Moore *et al*., (1995) [312] however, no mutations at these loci were seen in this study.

Several mutations in the *gyrA* gene that have been shown in other literature could not be identified during this test, as they were outside the range of the DNA selected for amplification, such as

those reported by Murakami *et al*., (2009) who documented substitutions in the *gyrA* gene at sites ranging from amino acid 62 to 158 [310]. Likewise, it is unknown if any isolates contained the Arg925His substitution that has previously been linked to fluoroquinolone resistance, or the 'QDNSV' amino N terminal extension discovered by Shetty *et al*., (2019) as the DNA amplification in this study did not incorporate these sections of the *gyrA* gene. In the future, expanding the range of the amplified site may be of use for identifying additional amino acid changes that are outside the QRDR. Alternatively, these may be detected using whole genome sequencing.

4.4.6 Comparison of Amino Acid Substitutions Between Paired Isolates

Some of the isolates taken from the antrum and corpus of the same patient were selected for *gyrA* sequencing as they had different antibiotic resistance MICs to levofloxacin when tested. The antrum and corpus isolates from patient 093 had a resistant MIC of 24µg/ml from the corpus biopsy (093B) and a susceptible MIC of 0.25µg/ml from the antral sample. However, no differences were found between the amplified section of the *gyrA* gene between the two isolates. This would benefit from further investigation using whole genome sequencing, as a direct comparison of the genomes

of the two isolates might help elucidate additional levofloxacin resistance mechanisms.

The isolates 255A and 255B had different amino acids present at position 63, however they both had the same resistance classification and MIC (0.25µg/ml). Isolate 255A contained a wild-type lysine residue at position 63, while 255B had a proline, which does not seem to have any impact on levofloxacin resistance. This amino acid substitution may indicate that the two isolates are from a *H. pylori* infection that began with one population that diverged slightly over time, as has been described previously [56].

For future studies, ideally it would be beneficial to acquire levofloxacin-resistant isolates from a bigger range of patients, as the paired isolates taken from individual patients in this study could be from the same *H. pylori* population and therefore not provide any additional information. However, the number of levofloxacin-resistant isolates available from the Nottingham Strain Collection was very limited. Furthermore, it has been established that divergent populations can exist within different niches of the stomach, and as such a genetic comparison of two isolates from the same patient might prove informative from this perspective.

4.4.7 Protein Structures and Modelling

Protein structure modelling was useful for identifying the location of where the levofloxacin resistance mutations sit spatially, and

helped provide insight into how the amino acids might influence levofloxacin binding. The predicted protein structures matched well with those that have been previously published [297], [344].

The modelling did not show any significant conformational changes in the tertiary structure of GyrA when comparing the different amino acid sequences. Previous molecular docking work has shown that for the most part, fluoroquinolone binding with GyrA is based on ionic bonding to amino acid side chains [297], [317], and so substitution of those specific amino acids (particularly 87 and 91) provides protection from fluoroquinolones, rather than a physical shape change of the protein. Additional molecular docking analysis of levofloxacin interactions with GyrA proteins comprised of different amino acid substitutions would provide valuable insight.

In the future, if further protein modelling work was to be performed it would be useful to obtain *gyrB* sequences from the isolates and construct a full DNA gyrase protein with the a2b2 subunit structure. Additionally, modelling of a larger range of amino acid sequences would provide more insight, particularly an 81 and 91 double mutation if one could be found, as well as the one containing the highly susceptible Thr87 substitution.

4.4.8 Strengths and Weaknesses

There was a limited number of levofloxacin-resistant strains in the Nottingham strain collection, which necessitated the selection of

some pairs of isolates from the same patient. However, it has been shown by Wilkinson *et al*., (2022) [56] that multiple genetically diverse strains of *H. pylori* may colonise different areas an individual's stomach, which is important to consider when deciding upon patient treatment. When comparing the results from E-test strips, some of the paired isolates that were selected for this study such as 093A and 093B had differences in MIC that put them into different resistance categorisations, which was of interest for further investigation. There is notable genetic diversity between *H. pylori* in the stomach between niches, and distinct subpopulations can be found [255]. Therefore, the selection of paired isolates from individual patients was beneficial not only for the sake of increasing the available number of levofloxacin-resistant isolates, but also to allow genetic comparison between populations from two areas of a patient's stomach. Additionally, obtaining isolates from other countries would be useful for a more in-depth analysis of *gyrA* mutations.

The laboratory strain NCTC 11637 was selected as a control strain during PCR amplification and sequencing. This strain has a measured MIC of 0.38µg/ml, which is classified as susceptible to levofloxacin based on EUCAST breakpoints, however, in the future, a different strain might be selected for this purpose that has a lower MIC. When trying to identify mutations that might cause small or nuanced changes in levofloxacin MIC, it would be useful to

have a more decidedly susceptible control strain for comparison's sake.

4.4.9 Conclusions and Recommendations

In conclusion, data in this study supports the argument that PCR amplification and sequencing can be powerful tools for identifying antibiotic resistance genes. In the case of levofloxacin resistance in *H. pylori,* amino acids 87 and 91 in the QRDR of the *gyrA* gene can indicate the antibiotic susceptibility status of the isolate. Of particular note are the mutations 87Lys, and 91Gly, Asn and Tyr which are all associated with resistance, and Thr87 which has been associated with very low levofloxacin MICs. Using PCR to detect these mutations is a quicker and more easily accessible method for determining resistance in *H. pylori* compared to culturing alone, and the genotypic resistance profile provided by PCR testing is comparable to phenotypic resistance levels. DreamTaq polymerases are sufficient for detecting resistance SNPs in the QRDR, and advances in PCR-related technologies are providing more diverse means for resistance gene detection.

This information may help clinicians provide patients with the most suitable treatment regimen. However, there are still questions that remain unanswered about the mechanism of resistance in *H. pylori* isolates such as 093B, which are strongly phenotypically resistant but did not contain any mutations in the quinolone resistance

determining region. Sequencing the entire genome may help elucidate what these resistance mechanisms are, to further safeguard antibiotic efficacy and appropriate use.

Chapter 5: Whole Genome Sequencing Techniques for the Detection of Resistance Polymorphisms

5.1 General Introduction

Antibiotic resistance in *H. pylori* is a growing concern, and infections with resistant strains can lead to treatment failure and the further spread of AMR. Testing for AMR via culture-based methods provides valuable information for clinicians, however, it is time-consuming and currently requires an endoscopy in order to obtain stomach biopsy samples for obtaining the bacteria. PCR-based methods are useful for quickly flagging up genetic markers in certain genes that are predictive of antibiotic resistance but are limited in scope to the target amplicons. Some cases have been reported of *H. pylori* isolates demonstrating phenotypic levofloxacin resistance with no discernible genetic cause in the target genes [300], [301], [302], [303]. Therefore, casting a wider analytical net may help identify additional genetic causes of resistance, in the form of whole-genome sequencing. A major source of antibiotic resistance is the mutation of the DNA that encodes the drug target protein, which modifies it in such a way that antibiotic binding is reduced or completely halted. Other means of antibiotic resistance

include the production of enzymes that can modify or break down the drug, something that blocks the target protein, or the blockage/removal of the antimicrobial compound from the bacterial cell altogether, and these can be more difficult to pinpoint during PCR amplification and Sanger sequencing approaches.

H. pylori has a high rate of genetic mutation, even among bacteria [345], and can also acquire resistance genes through a number of processes. The quantity of resistance genes and the associations between them are a hurdle for determining phenotypic resistance, however whole genome sequencing (WGS) may prove useful for predicting the presence of AMR, as well as detecting virulence factors such as the *cag*PAI and *vacA* isotype [345]. Whole genome sequence data can be stored in repositories that are accessible worldwide and can be used to help identify trends in resistance, and genome-wide interactions between different genes that go unnoticed when analysing individual genes. The data can be retroactively interrogated in the future based on new discoveries and using new analysis techniques. Using WGS on a selection of the isolates from the Nottingham Strain Collection in combination with the susceptibility data already acquired can provide important insights into antibiotic resistance, and aid future understanding.

5.1.1 Antimicrobial Resistance Genes Outside of Target Proteins

The cause of antibiotic resistance within bacterial populations is not limited to the presence of SNPs within specific genes, which tend to only impact the target proteins of specific antibiotics. There are also other broader mechanisms such as efflux pumps and the production of biofilms which can provide general defences against a broader range of antimicrobial agents. There are also cases where two or more genes act synergistically to produce a greater resistance phenotype than one gene alone. In these cases, there may be several genes responsible for phenotypic resistance, and PCR testing for the presence of a mutation within an individual gene may not be successful in predicting antibiotic resistance within an isolate. Whole genome sequencing can be used for identifying multiple different types of antibiotic resistance gene at the same time, along with other genes that can modify how the organism can survive attempts at eradication therapy.

5.1.2 Epistatic Mutations

Epistatic mutations (or compensatory mutations) describe the interaction of two or more genes that can modulate the fitness of an organism. The genes do not necessarily need to be close together on the genome to interact with one another. In the case of antibiotic-resistance genes, epistatic mutations can help offset or

compensate for the fitness cost that is often associated with AMR. This means that a mutation that can confer levofloxacin resistance but would otherwise be deleterious to the bacterium can still be present in the genome, and the negative side effects are negated. This effect has been suggested to occur in *H. pylori*, in relation to clarithromycin resistance [346]. In Björkholm *et al*. (2001), clarithromycin-resistant strains of *H. pylori* were shown to be generally less fit than susceptible strains, as evidenced by resistant populations being outcompeted by susceptible bacteria in mouse models. However, further comparison of resistant isolates demonstrated a loss of this fitness cost in subsequent generations, which may indicate the development of a compensatory mutation.

5.1.3 Horizontal Gene Transfer

Communities of bacteria can transfer genes between themselves via several methods, such as conjugative transfer of plasmids between two cells, phage moving DNA from one cell to another, and bacteria taking up free DNA from the environment via transformation [347]. In *H. pylori*, the conjugation-like transfer of plasmids has been noted, and plasmids are reportedly found in around 50% of strains [348]. Although the most common resistance mutations in *H. pylori* are found on the chromosome [300], [343], plasmids can still protect against antibiotics (for instance, by increasing biofilm production [349]). Natural

transformation is common in *H. pylori*, which involves taking up foreign DNA from the environment and incorporating it into the genome [348]. *H. pylori* has been described as 'naturally competent', as it can take up exogenous DNA during normal growth conditions without the need to modulate environmental conditions to induce competence [350]. The captured DNA is incorporated into the genome via homologous recombination, which has been shown to contribute to the spread of AMR genes within and between microbial populations [351]. Exogenous gene integration, along with the hyper-mutable nature of *H. pylori* genes means that resistance genes can be acquired or developed in new loci. WGS can be used to identify these [345].

5.1.4 Efflux Pumps

Efflux pumps are membrane proteins that can remove antibiotics from the bacterial cytoplasm and prevent them from reaching an effective concentration [352]. *H. pylori* has been found to contain a range of genes encoding efflux pumps, some of which have been linked with clarithromycin resistance [353]. The enzyme SpoT has also been linked to multi-drug resistance in *H. pylori* by upregulating the efflux pump Hp1174 (encoded by the *gluP* gene) and is also linked to biofilm formation [354]. Outer membrane proteins (OMPs) and porins have also been linked to resistance. Clarithromycin-resistant *H. pylori* isolates have been associated

with up-regulation of transmembrane proteins HopT and HofC, as well as the porin family protein OMP31 [355].

5.1.5 Biofilm Formation

Some bacterial populations have the ability to create biofilms, which are sticky protective layers surrounding the cells formed of secreted polysaccharides, proteins, and extracellular DNA [356]. Biofilms can cause decreases in antibiotic effectiveness of 10-1000-fold, as the drug molecules are unable to permeate the biofilm layer and antibiotics cannot reach a high enough concentration to be effective. They also provide a reservoir of extracellular DNA that can aid the spread of resistance genes [357]. In *H. pylori*, biofilm formation is regulated by a slew of genes that play roles in polysaccharide production, motility, and quorum-sensing signalling molecules [356]. *H. pylori* Isolate 444A from the Nottingham Strain Collection has been reported as having high biofilm-forming ability, and interrogation of the genome using WGS has been used for investigating the presence of biofilm-associated genes [349].

5.1.6 Duplicated Genes Within the Genome

In many instances, bacteria can have more than one copy of a particular gene on their chromosome, especially for genes that are essential for survival or require high levels of gene expression. Having multiple copies of a gene can increase fitness and can also drive the development of resistance due to the increased likelihood

of at least one copy of the gene acquiring a mutation. When trying to predict phenotypic resistance from a genome sequence, it is useful to know how many copies of the duplicated gene need to contain resistant mutations to provide overall protection to the bacterium. For example, *H. pylori* has two copies of the 23S rRNA gene, each of which can have different mutations, including SNPs that confer resistance to clarithromycin [358].The impact that one or both having resistance mutations have on phenotypic resistance is still disputed in *H. pylori*, with some literature stating that it is likely that dual mutations are needed for resistance to be expressed phenotypically [300], while others have found that isolates that contain even a single resistant copy of the gene can cause phenotypic resistance [359]. It has been demonstrated in other bacteria such as *Streptomyces ambofaciens* [360] and *Neisseria gonorrhoeae* that additional mutated copies of the 23S gene can be linked to higher levels of macrolide resistance [361]. It is difficult to tell from PCR amplification if and how many copies of a gene a bacterial species may contain, and of those how many contain AMR mutations. However, WGS can be used to detect the number of gene copies, and what proportion of those genes carry resistance mutations.

5.1.7 Whole Genome Sequencing Overview

Sequencing technologies have been constantly evolving over the past few decades, developing from an extremely expensive and time-consuming process [362] into something that can be performed by an individual using a laptop, even when working in a remote glacial location [363]. Sanger sequencing (as described in the previous chapter) is a cheap and reliable method for obtaining short sections of DNA sequences. However, this platform has its drawbacks, and sequencing entire genomes using Sanger sequencing would be extremely inefficient [364], as this method involves sequencing an individual DNA fragment at a time per reaction. Whole genome sequencing can be performed using next-generation sequencing (NGS), which is characterised by a much higher throughput of data because multiple DNA molecules can be sequenced in parallel [364]

There are several different NGS platforms available, such as Illumina, single-molecule real-time (SMRT) sequencing by Pacific Biosciences (PacBio) [365], and Oxford Nanopore Technology (ONT) [366]. PacBio SMRT sequencing and ONT sequencing both rely on long individual DNA molecules for sequencing, while Illumina is classed as a short-read sequencing type [366]. It is also by far the most predominantly used form of WGS, although long-

read sequencing has become more popular over recent years, both alone and in combination with short-read sequencing [367].

5.1.7.1 Illumina Sequencing

To perform Illumina sequencing, the template DNA is first prepared by fragmenting it into small ~350bp segments, with adapter sequences ligated to both ends [368]. The sample is then loaded onto an Illumina flow cell, which is covered with primers that are complementary to the adaptors on the DNA fragments. Both ends of each sequence bind to the cell, forming a bridge structure. Free nucleotides within the sample are used to create the complementary strand to the DNA bridge, and then a denaturation step releases one end of the bridge, leaving the other anchored to the substrate. This process is repeated several times, resulting in clusters of identical sequence fragments on the flow cell membrane, in what is known as the bridge amplification cycle. Fluorescently labelled nucleotides are then incorporated one at a time onto the complementary template strand, with each of the four bases emitting a different colour. Based on the colour emitted by the sequence clusters during each nucleotide incorporation, the sequence of each fragment can be determined and then later assembled into a full genome, as shown in **Figure 31** [366], [369].

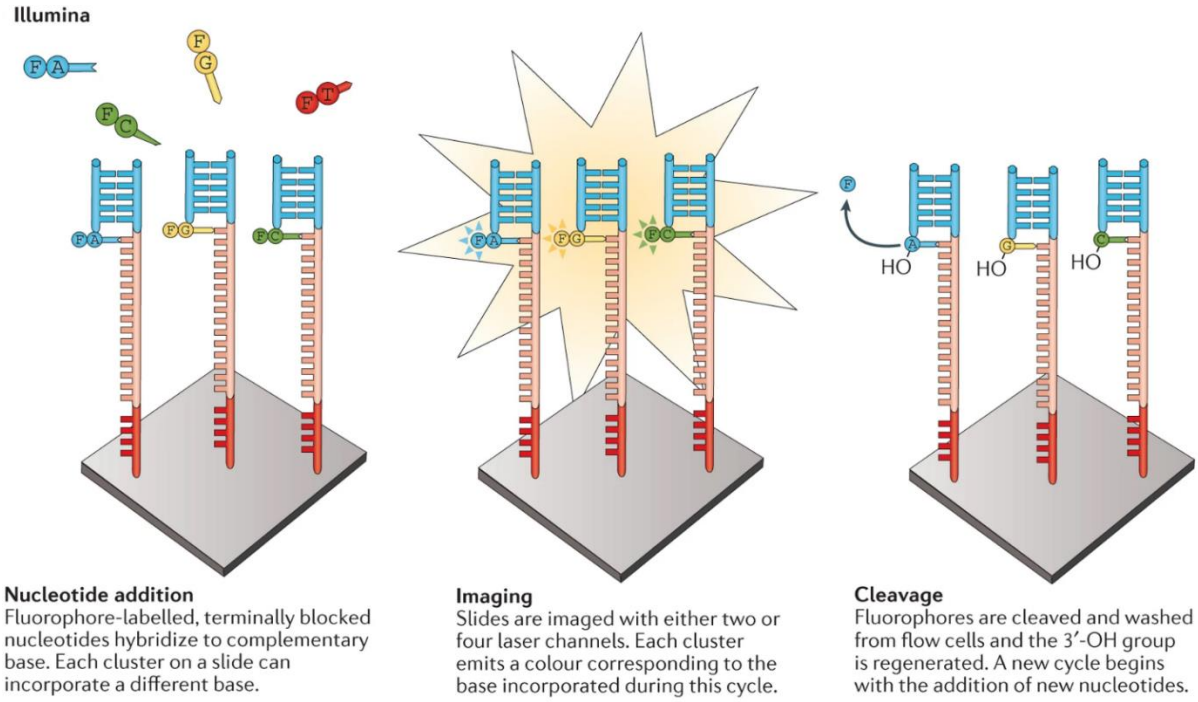


Figure 31 *- The process of Illumina sequencing via synthesis.*

Fluorescently labelled nucleotides are added to the flow cell and bind one at a time to the complementary base on the target sequence. After each new nucleotide is bound, lasers excite the fluorescent tag, which emit different wavelengths of light for each of the four nucleotide bases, and a detector registers the fluorophore signal for each cluster of sequences. The fluorophores are cleaved and removed, ready for the next base to be sequenced. Image adopted from Goodwin et al., (2016) [366].

The process of Illumina sequencing provides extremely high coverage of the DNA sequence. Coverage refers to the number of times an individual nucleotide is represented in the sequencing data. It is usually represented as an average value of the theoretical coverage across the sequenced genome given the length of the target sequence and the number of nucleotides sequenced, although this figure is an estimate based on the

assumption that the reads are evenly distributed along the length of the template sequence [370]. The more depth of coverage a target region has, the less likely it is for an error to appear in the consensus sequence, as even if one read contains an incorrect base call, several others that cover the same location can be used to correct it (**Figure 32**). Illumina sequencing generally has the best coverage and provides sequences with a low error rate compared to long read sequencing methods [370].

This comes with certain drawbacks, however. Illumina sequencing requires a fragmentation of the target sequence, which obscures epistatic links between genes where mutations earlier in the sequence alter the expression of a downstream gene [371]. Further problems occur during assembly. Multiple short reads means that duplicated genes or repetitive sequence regions cannot be confidently placed in the correct context. In the case of *H. pylori*, duplicated resistant copies of the 23S rRNA gene may be linked to increased levels of clarithromycin resistance [358], but differentiating resistant and susceptible copies would be difficult using Illumina data. Repetitive regions may also lead to truncation of genes, or gaps in the assembly [370].

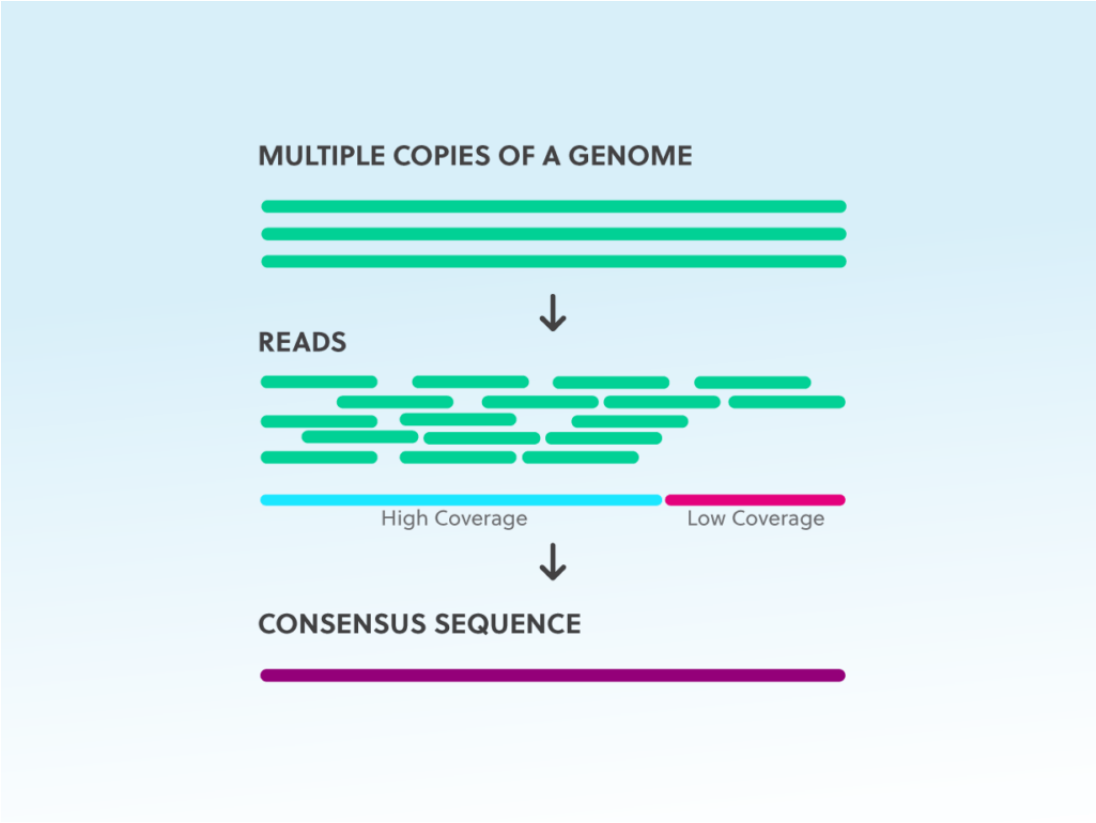


Figure 32 *- An illustration of sequencing depth of coverage.*

Regions that have high sequencing coverage can be assembled into a consensus with a high degree of confidence, whereas areas with low coverage may contain sequencing errors that enter the assembly sequence uncontested by other reads.
Image taken from iRepertoire Inc (irepertoire.com) [372].

5.1.7.2 Nanopore Sequencing

Nanopore sequencing was largely developed by Oxford Nanopore Technologies and utilises MinION flow cells, which are portable sequencers that can be connected to any computer. The transportable nature of this sequencing technology has boosted the popularity of the MinION due to the versatile locations it can be used in. Nanopore sequencing works by sequencing long individual strands of DNA without an amplification step like in Illumina

sequencing. Sequencing adaptors are attached to the double stranded target DNA molecules, which can vary from kilobases to megabases in length [373]. The MinION flow cell consists of a lipid membrane that contains thousands of protein nanopores, each forming a channel from one side of the membrane to the other. As the DNA strand moves across the electrically charged membrane through the pore, each of the four bases disrupts the current across the membrane by a set amount due to their individual chemical structures. This disruption of the current is detected and can be used to determine the order of the bases that passed through the pore. From this method, very long individual strands of DNA can provide high volumes of sequencing data [374].

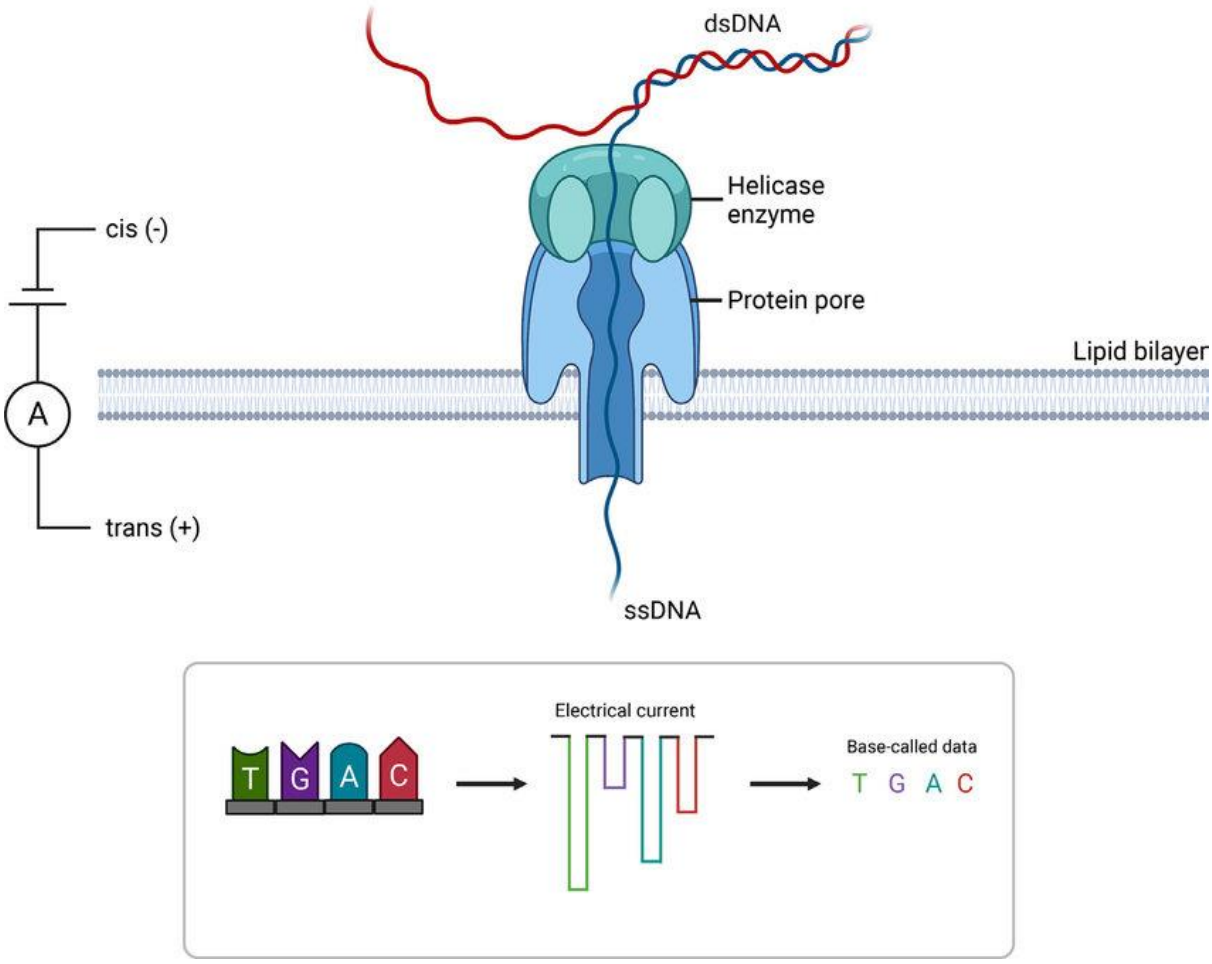


Figure 33 *– A diagram demonstrating the principles of nanopore sequencing.*
An electric current is applied across the lipid bilayer membrane, which has protein nanopores embedded in it. A strand of DNA is captured and unwound by the helicase enzyme. The negatively charged DNA is drawn through the pore, and as each base passes through, the electric current is disrupted by a different amount depending on if the base is T, G, A or C. Detecting these changes in the current allows for the sequence to be recorded. Image taken from Beckett et al., (2021) [375].

There are several benefits to a long-read sequencing approach compared to Illumina sequencing. The length of the individual DNA molecules being sequenced can preserve repetitive sequences accurately and can reduce the likelihood of gaps being present in the final assembly. Duplicated genes can be conserved *in situ* with

easy identification of any genotypic differences between copies, and the portability of the MinION flow cell is also an asset, particularly for sequencing in remote locations such as glaciers [363] and rainforests [376], so delicate samples do not need to be preserved and transported. Ease of transport and setup is useful for quickly gathering sequencing data in the case of quickly mutating pathogens such as the SARS-CoV-2 virus [375].

There are, however, drawbacks to nanopore sequencing. The error rate compared to Illumina is high, with an accuracy of around 92%. While this is acceptable for many applications, the detection of resistance single nucleotide polymorphisms relies on high accuracy. An erroneously sequenced base may imply an antibiotic resistance mutation is present, and conversely, an SNP might be missed [374]. Additionally, if a sequence has more than three of the same base consecutively, the error rate increases due to the difficulty in distinguishing individual bases of the same type as they travel through the nanopore [366]. Unfortunately, the process for procuring sufficient quantities of high molecular weight DNA can require careful and labour-intensive work in order to preserve DNA strand length and get the benefits from long-read sequencing.

5.1.7.3 Pacific Bioscience

PacBio is another long-read sequencing platform, but the error rates associated with long read sequencing are reduced by using

circular consensus sequencing (CCS), which can result in very high accuracy reads (99.8%). This functions by forming hairpin loops at both ends of the double-stranded sequence of interest, allowing several sequencing passes of the template DNA to occur as if repeatedly sequencing a circle of DNA. The repeated reads are then combined to gather a highly accurate consensus [377]. However, PacBio sequencing can be significantly more expensive than other sequencing types (**Table 19**) which is a barrier to frequent usage [349].

Table 19 *- A comparison of three whole genome sequencing platforms; Illumina, Oxford Nanopore Technologies, and Pacific Biosciences.*

Sequencing Platform	Sequencing Reaction	Read Length	Relative Coverage	Relative Cost
Illumina	Sequencing by Synthesis	Short	High	Low
Oxford Nanopore	Detection of current that varies as each base passes through a pore	Long	Low	Low
Pacific Biosciences	Sequencing by Synthesis	Long	Low	High

Processing and interpreting the WGS data requires knowledge of bioinformatics analysis, which is currently a barrier that prevents use of this technology for general diagnostic use in clinical settings. However, in recent years online tools and repositories such as Galaxy [378] and Geneious [379] have been developed which provide storage and analysis for large quantities of genomic data,

so limited computing power and local memory are not significant obstacles. Analysis software and programs have also been simplified and equipped with user interfaces to allow for more widespread use, and no longer require coding knowledge for at least general tasks. Currently, it is still beneficial to have in-depth computing and coding knowledge for more complex bioinformatic analysis, particularly for the development of new tools and techniques, as well as for solving problems that are outside the scope of available tools. That said, the accessibility of analysis tools available for the processing of long-read sequencing data has been improving in recent years and is likely to continue improving [380].

5.1.7.4 Genome Polishing

The benefits and drawbacks of each type of WGS mean that no single one is perfect for searching for AMR genes, however, it is possible to perform a combination of long and short-read genome assembly that helps mitigate some of the issues. Using ONT and Illumina sequence data together in a hybrid approach can allow for the benefits of long read data for the detection of gene duplicates and repetitive sequences, but the higher read accuracy of short read data for SNP detection. This can provide the benefits of the PacBio sequencing approach but at relatively more affordable rates [349], [381].

Polishing is the correction of errors that may have made it to the genome assembly by using long and short reads to resolve incorrect base calls and genome mis-assemblies [382]. The reads are aligned back against the assembly in one or more iterations to produce a corrected sequence, although care should be taken not to introduce new errors at this stage with poor-quality reads [383]. The extent of the polishing and correcting depends on the tools and the settings used. Several tools are available for genome polishing, such as Medaka [384] and Racon (Rapid Consensus) [385], which vary based on the algorithms used for balancing polishing speed and accuracy. Racon functions based on the partial order alignment graph principle [386], while Medaka (developed by ONT) uses a neural-network-based approach for selecting the most likely sequence in the alignment. The various polishing tools can be used individually or in combination to achieve the necessary results for a project. Polished long-read genomes have been shown to have significantly improved accuracy compared to unpolished ONT assemblies, which is a useful asset for the detection of SNPs [382]. However, performing multiple rounds of polishing can be a time consuming and labour-intensive task. Careful consideration is needed when balancing workload vs the increase of accuracy, as continuous genome polishing may produce decreased returns on improvement after a certain point and could even be deleterious to genome quality if performed incorrectly [383].

5.1.8 Antibiotic Resistance Gene Databases

Once the genome sequence has been acquired, mutations and resistance genes need to be identified. There are some databases and tools available that can aid with identification of common resistance genes, such as CARD (The Comprehensive Antibiotic Resistance Database) [387], MEGAres [388], and ResFinder [389]. These tools can identify resistance genes present within a sequence but are limited to those that have already been identified and deposited into the repository. Unfortunately, *H. pylori* is often underrepresented in such databases, and they rely on being manually updated and maintained [390]. This process requires researchers detecting and reporting *H. pylori* AMR genes more frequently, to build up resources for the future.

5.1.9 Summary and Aims

H. pylori antibiotic resistance is a widespread problem, and detection of AMR genes and resistance mechanisms can be limited when only performing PCR amplification of specific genes and Sanger sequencing. Whole genome sequencing can provide important insights into other resistance mechanisms such as biofilms, epistatic mutations, and repeated genes. The method of sequencing can impact detection of resistance genes. Long read sequencing methods such as ONT have low coverage and are error prone, but preserve the context of the genome sequence, while

Illumina sequencing has a much higher sequencing depth but must first be fragmented into small sections, which obscures genome features. Performing genome polishing can help to moderate these issues and provide a high-quality genome with repetitive gene sequences intact, and few errors. However, this can be a time-consuming and complicated task, and if done incorrectly can even introduce additional errors into the sequence. Online databases such as CARD can then be used for identifying a range of resistance genes within the genome assembly, although they are generally not optimised for *H. pylori* use.

As such, in this chapter the main aims are as follows:

1. **Acquire whole genome sequence data of a range of *H. pylori* isolates from the Nottingham Strain Collection using both Illumina and Oxford Nanopore Technology sequencing types and perform bioinformatic analysis of both sets of data.**

2. **Complete genome assembly of the sequencing reads and perform genome polishing. Compare genome quality before and after polishing to ascertain if a polishing step can aid identification of AMR genes.**

3. **Use the online resistance gene database CARD to identify any AMR genes in the genome assemblies and compare the results to previously obtained phenotypic resistance data to assess database suitability for use with *H. pylori.***

5.2 Methods

5.2.1 DNA Extraction and Purification

14 *H. pylori* isolates were chosen for whole genome sequencing, including laboratory strain *H. pylori* NCTC 11637 as a control. Of these, 7 isolates had an MIC classifying them as resistant to levofloxacin, 4 were classified as 'borderline susceptible' and 3 were classified as susceptible. Genomic DNA extraction and purification was performed as described in Section 4.2.3. The genomic DNA sample requirements for Oxford Nanopore Technology (ONT) sequencing was 100µl sample volume at a concentration of 60ng/µl, which was checked using a SimpliNano ™ Nanodrop (Biochrom). Precautions were taken as previously discussed to reduce DNA shearing and maintain a high molecular weight.

5.2.2 Sequencing

Sample preparation and genome sequencing was performed at Nottingham Trent University by Dr Jonathan Thomas and his team, using Oxford Nanopore and Illumina MiSeq. Oxford Nanopore sequencing was performed using minION with pore type R9.4.1. Basecalling of the .fast5 files was performed using the Guppy (v6.2.1) Super High Accuracy model, which gave the outputs as fastq.gz files. The sequence files were analysed at the University of

Nottingham. One levofloxacin-resistant isolate, 613A, was removed from the study at this point due to contamination.

5.2.3 Data Quality Control and Assembly

For ONT sequencing, the data received included a compressed fastq.gz file for each sample. The Illumina data was paired-end, and contained 2 fastq.gz files for each sample, 'R1' and 'R2'. All files were uploaded to the online genomic analysis platform Galaxy at usegalaxy.eu [378]. A published whole genome sequence of *H. plyori* laboratory strain NCTC 11637 (GenBank Accession Number LS483488) was also uploaded to Galaxy for use as a reference. Initial quality checks were performed on the sequencing data using FastQC Read Quality Reports (Galaxy Version 0.74+galaxy1) [391], and results aggregated for viewing using MultiQC (Galaxy Version 1.24.1+galaxy0) [392]. Porechop (Galaxy Version 0.2.4) [393] was used to detect and remove any nanopore sequencing adapters left in the ONT data. Further quality control of the ONT reads was performed using Nanoplot (Galaxy Version 1.43.0+galaxy0) [394]. The tool Fastp (Galaxy Version 0.24.0) [395] was used for quality control of the Illumina paired-end reads, to filter out reads that were too short (less than 15bp) or low-quality (Q of less than 15) and to remove any remaining adapter sequences. Fastp also provided quality reports from before and after filtering.

Three of the isolates for the nanopore sequencing required two passes of minION sequencing due to low DNA concentration in the samples. In these cases, the nanopore flow cell was washed after the initial run, and the three samples reloaded and sequenced again to provide as much sequencing data as possible. This second set of .fastq data was also uploaded to Galaxy, and the Run 1 and Run 2 data for each isolate were combined together using the tool 'Concatenate Datasets tail-to-head (cat)' (Version 09.2018) [396]. The concatenated files were then run through Filtlong (Galaxy Version 0.2.1) [397] to filter the .fastq file to a total of 500 million base pairs, sorted by the quality of the reads. This was to ensure that the large, concatenated file size could be reduced enough to be compatible with downstream Flye assembly. De novo assembly of ONT reads was performed using Flye (Galaxy Version 2.9.5) [398], with polishing iterations set to 1. The assembly for isolate 733B would abort with these parameters due to disjointigs failing to assemble, therefore the Flye setting 'perform metagenomic assembly' was selected in order for the assembly to be completed on this sequence.

A QUAST (Quality Assessment Tool for Genome Assemblies) (Galaxy Version 5.3.0) [399] report was run on the Flye Assembly output to check the number of contigs, the length, and the N50 data. Fasta Statistics (Galaxy Version 2.0) [400] was used to

provide summary statistics on the consensus read, and Bandage Image [401] was used to visualise the assembly.

5.2.4 Genome Polishing

Correcting (or 'Polishing') of the ONT long read Flye contigs was performed using both the raw ONT long reads and the R1 short read of the Illumina sequencing data. The methodology for Long Genome Polishing was provided by Dr Daniel Whiley and Galaxy Training Materials [402], [403]. The long-read polishing was performed four times using the tool Racon (Galaxy Version 1.5.0) [385] for error correction. One additional round of long read polishing was also performed using Medaka Consensus Pipeline (Galaxy Version 1.7.2) [384], a tool that uses neural networks to compare sequences against draft assemblies and polish them. Additionally, two more rounds of Racon polishing were performed using the short-read R1 Illumina sequences.

The polished consensus FASTA sequences were extracted, or in the case of assemblies with more than one contig, the longest contig was selected and extracted. These were then analysed for antibiotic-resistance genes using the Comprehensive Antibiotic Resistance Database (CARD) for assessing antibiotic resistance of the assembled sequences [387].

5.2.5 Assembly Annotation and Core Genome Alignment

The contig .fasta files of isolates 93A and 93B (previously labelled 93C) had been kindly made available by Dr. Daniel Whiley, who had previously performed ONT sequencing on a selection of the Nottingham Strain Collection isolates. The sequence data for 93A and 93B were included in the following analyses. The prokaryotic genome annotation tool Prokka (Galaxy Version 1.14.6+galaxy1) [404] was used with default settings on the assembly files to produce .gff3 annotation files. Annotation is the process of assigning labels to genes and other features of the DNA sequence.

The collection of Prokka .gff3 files were then used with the core gene alignment tool Roary (Galaxy Version 3.13.0+galaxy3) [405]. Roary produced three outputs, a Gene Presence Absence .csv table, a Core Gene Alignment .fasta file, and a summary statistics table. The Core Gene Alignment .csv table was used to create a maximum likelihood phylogenetic tree using RAxML (Galaxy Version 8.2.12+galaxy1) [406]. Using the Roary Core Gene Alignment .csv file, the Prokka .gff3 annotation file, and a metadata .csv table (produced using Microsoft Excel) of the antibiotic resistance profiles of each of the samples, the online visualisation tool Phandango [407] was used to create an image that showed the relationship between the isolate phylogeny and

antibiotic resistance data, as well as the corresponding core genome data.

5.3 Results

5.3.1 *H. pylori* Sequencing Initial Quality Control

All sequencing data was uploaded to Galaxy.eu, where an initial check of the sequencing quality was performed to ensure no isolates needed to be re-sequenced before continuing with the assembly pipeline. FastQC Reports provided graphs displaying per base and mean sequence quality, and read length distribution, all of which appeared as expected for this sequencing run. The N content, sequence duplication levels, and overrepresented sequence data were all near zero, indicating that the sequences could be used for downstream assembly purposes. The GC% content was at 38-43% for all isolates, which is flagged by FastQC Report as a low value, however, this range is typical for *H. pylori* [408] and served as an indicator that the correct organism had been sequenced. The FastQC output for the ONT sequencing data for isolate 286A showed a normal GC distribution across most of the sequence length, but then showed a long tail sequence consisting of almost 100% GC bases. This tail is likely an artefact from the sequencing process and was removed during trimming and filtering.

5.3.2 Illumina Sequencing Quality and Statistics

Further qualitative data was gathered about the Illumina sequences using fastp. The average quality score (Q value) of all the Illumina reads ranged from 34.1-36.4, with a mean Q score of 36. A Q value of ≥30 had been selected as a quality benchmark for reads as it equates to 99.9% read accuracy [409], and any reads below Q15 were removed. Over 90% of bases for 11 out of the 12 sequencing runs were of Q30 or higher, with only sample 934A having fewer than 90% of bases of quality Q30 (89.62%) (**Table 20**). The sequencing data from isolate 875A had the highest percentage of reads over Q30 (95.85%), and 97.03% of reads over Q20. Reads shorter than 15bp in length were filtered out from the Illumina data as they are likely to map incorrectly during genome assembly. The number of filtered-out short sequences varied from 0% of the sequencing data (11637) to 24.38% (isolate 934A). The total number of reads per sample ranged from 1235014 to 2125722, with an average of 1707106.3 reads. The average GC value for the Illumina reads was 40.9%.

***Table 20** - A table of read quality statistics for raw Illumina sequencing data, obtained from H. pylori DNA.*

Isolate	Mean length before filtering (R1, R2)	Total no. reads	Total no. bases	Q20 bases	Q30 bases	GC content	Removed short reads (<15bp)
256B	226bp, 226bp	1709832	387710869	37172344 (95.88%)	365332388 (94.23%)	40.72%	42770 (2.50%)
875A	190bp, 190bp	1693480	322370417	312797184 (97.03%)	308977581 (95.85%)	40.48%	200202 (11.82%)
935B	209bp, 210bp	1585816	333232365	298633555 (89.62%)	287403966 (86.25%)	41.11%	169082 (10.66%)
201A	231bp, 231bp	1784162	413138622	399483132 (96.69%)	393802913 (95.32%)	40.37%	38266(2.14%)
11637	183bp, 183bp	1676378	307688256	292754299 (95.15%)	287617812 (93.48%)	40.23%	0 (0.0%)
642A	221bp, 222bp	1611930	357992273	32996004 (92.17%)	319960742 (89.38%)	41.07%	100406 (6.22%)
733B	236bp, 236bp	1504660	355816562	342754928 (96.33%)	3373308 (94.8%)	40.52%	19784 (1.31%)
201B	185bp, 185bp	1868078	347167055	334300011 (96.29%)	329438645 (94.89%)	40.77%	283462 (15.17%)
286A	215bp, 216bp	2125722	459072680	443944573 (96.7%)	437633645 (95.33%)	40.99%	153482 (7.22%)
642B	204bp, 204bp	2025660	414590999	39859737 (96.14%)	392425506 (94.65%)	40.71%	135518 (6.69%)
733A	226bp, 226bp	1642102	372504117	357387457 (95.94%)	350818173 (94.18%)	42.78%	77488 (4.71%)
875B	194bp, 194bp	1729548	336465235	325774814 (96.82%)	321576314 (95.57%)	40.75%	182590 (10.55%)
934A	166bp, 167bp	1235014	206055348	193982075 (94.14%)	189937879 (92.18%)	41.13%	301176 (24.38%)

Data was acquired using fastp and MultiQC analysis. Q20 and Q30 indicates the number and percentage of bases with a quality score of 20 or more, or 30 or more respectively. R1=Illumina Read 1, R2=Illumina Read 2, bp=base pairs.

5.3.3 Oxford Nanopore Technologies Sequencing Quality and Statistics

Using NanoPlot, in-depth information for the ONT long-read sequences was obtained, shown below in

Table 21. The N50 value among the sequences ranged from 1341 (isolate 642B) to 11195 (isolate 11637), with an average N50 of 5217.2. The N50 value is a measure of sequence size distribution. Compared to the total assembly length (in the case of *H. pylori* WGS, around 1.6 million bp), if all the sequencing reads are arranged in size order from largest to smallest, a certain number of reads will be needed to make up 50% of the genome length (0.8 million bp). The size of the smallest read that makes the genome up to the 50% length is the N50 value, i.e. half the total genome sequence length can be made up of reads that size and larger.

The mean quality ranged from 10.9 to 11.4, with an overall average of 11.2. A Q score of >10 is considered an acceptable benchmark for ONT sequencing data [410]. The sequence data for isolates 642A and 642B both had the lowest mean quality value of 10.9, while sequences 875A, 935B, 733A and 733B all had the joint highest quality value. All of the sequences had more than 95% of reads with a Q value of 10 or more, however, less than 3% of reads from each sequence were above a Q15 value. The sequence data from isolate 11637 had the highest number of reads (787414bp), the highest mean read length (5652) and the highest N50 value (11195), as well as the longest individual read (90,668bp). Isolate 875B had the single highest quality read, with a 102bp read of Q26.5.

***Table 21** - Quality control statistics of Oxford Nanopore Technologies sequencing data.*

Isolate	No. Reads	No. Bases	Mean Read Length	n50	Mean Quality	Longest Read (Q Value)	Highest Q Read (length)	Reads >Q10	Reads >Q15
256B	131932	515521698	3907	6276	11.3	48822 (10.4)	20.9 (770)	128372 (97.3%)	1798 (1.4%)
875A	401114	819406872	2042	2943	11.4	40627 (10.5)	21.7 (247)	389427 (97.1%)	11508 (2.9%)
935B	426209	873340044	2049	2727	11.4	42228 (12.3)	21.1 (471)	414874 (97.3%)	10324 (2.4%)
201A	76073	247158267	3249	5319	11.2	33844 (10.9)	19.1 (675)	73750 (96.9%)	752 (1.0%)
11637	787414	4450600194	5652	11195	11.2	90668 (11.4)	29.3 (9)	763538 (97.0%)	11776 (1.5%)
642A	283448	675542273	2383	3023	10.9	43716 (12.5)	20.2 (598)	271073 (95.6%)	1775 (0.6%)
733B	495168	1416446554	2860	4281	11.4	48956 (10.3)	21.7 (777)	482230 (97.4%)	11908 (2.4%)
201B	38323	160514398	4188	5897	11.2	33227 (12.4)	19.1 (258)	37198 (97.1%)	301 (0.8%)
286A	430440	573941143	1333	1356	11.3	66963 (15.7)	20.3 (917)	417048 (96.9%)	8661 (2.0%)
642B	704534	915112647	1298	1341	10.9	32632 (13.2)	21.1 (193)	671213 (95.3%)	5255 (0.7%)
733A	115253	617893984	5361	9398	11.4	63425 (12.4)	21.6 (289)	112444 (97.6%)	2069 (1.8%)
875B	832935	3055083480	3667	5648	11.2	55160 (10.0)	26.5 (102)	806550 (96.8%)	12014 (1.4%)
934A	88633	485519866	5477	8420	11.2	62952 (10.2)	20.3 (241)	86179 (97.2%)	689 (0.8%)

Statistics acquired using Nanoplot. Read lengths are given in base pairs.

In comparison, the mean read length of the ONT sequencing reads were 16 times higher than the Illumina reads, and the average quality score of the Illumina reads was 3 times higher than that of the ONT sequencing reads, albeit on a logarithmic scale (**Table 22**).

***Table 22 -** Comparison of Average Quality Control data of Illumina and Oxford Nanopore Technologies Sequence Data.*

	Illumina Sequencing Statistics		ONT Sequencing Statistics	
	Range	**Average**	**Range**	**Average**
Number of Reads	1235014 to 2125722	1707106.3	38323 to 832935	370113.5
Number of Bases (bp)	206055348 to 459072680	354908061.4	160514398 to 4450600194	1138929340.0
Mean Read Length (bp)	166 to 236	206.6	1298 to 5652	3343.5
Average Quality Score	34.1 to 36.4	36.0	10.9 to 11.4	11.2

Data obtained from raw sequencing reads before filtering or trimming. Illumina quality control data obtained using fastp, ONT quality control data obtained using NanoPlot. bp=base pairs, ONT = Oxford Nanopore Technologies.

5.3.4 Porechop Removal of Oxford Nanopore Technologies Sequencing Adapters

When removing ONT sequencing adapters from the long read data using Porechop, Barcode 01 (isolate 256B) repeatedly caused Porechop to receive a fatal error code and cease operating. Dr. Jonathan Thomas who had performed the initial sequencing at Nottingham Trent University identified and removed one .fastq file within the Barcode 01 dataset that was causing Porechop to fail for unknown reasons. After removal, Porechop was able to complete the operation successfully. This meant that the Barcode 01 dataset contained 4000 fewer reads than before the .fastq file removal, however, there was still enough sequencing data to perform a full assembly.

5.3.5 Initial Short and Long Read Assembly Comparisons

Two initial *de novo* assemblies were constructed using the long and short-read sequencing data for isolate 201A, to assess and compare assembly completeness (**Figure 34**). Both assemblies were assessed for quality using Quast and compared to the *H. pylori* NCTC 11637 reference genome. The Illumina short read data was assembled using Shovill, and the resulting assembly contained 44 contigs over 100bp in size (contigs smaller than this were not included in the Quast analysis), which ranged in size from 150bp to

345,182bp in length. The assembly formed a total length of 1,321,569 bp, representing 78% of the length of the reference genome. The assembly contained many unresolved or branching pathways and several contigs that did not integrate into the sequence. The ONT long reads were assembled using Flye into a single circularised contig of 1,617,176bp, which represents 97.4% of the length of the reference genome.

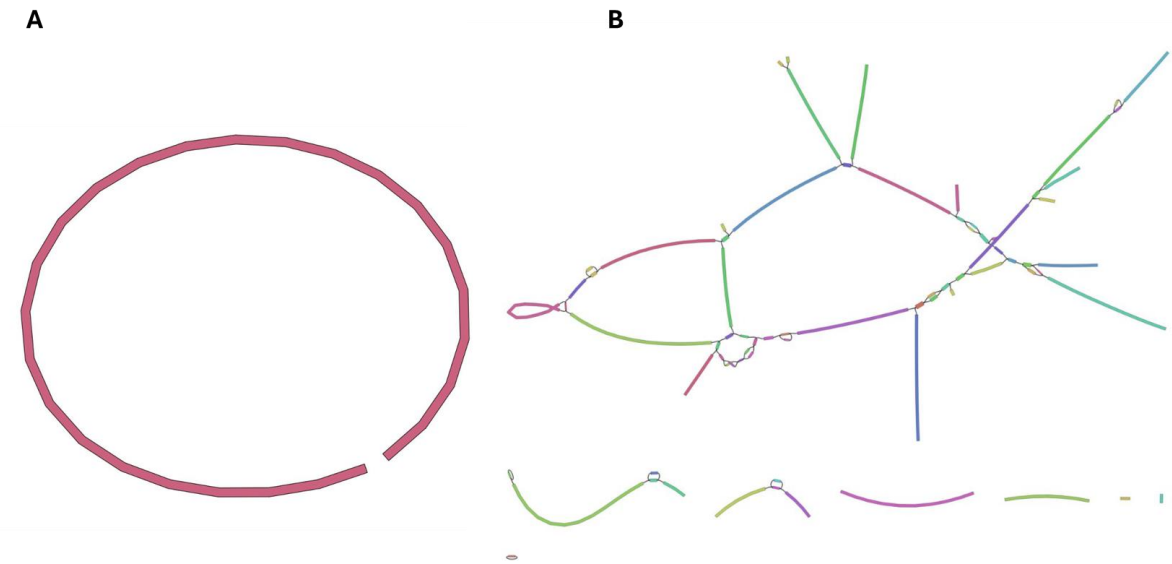


Figure 34 - *A comparison of two whole genome assemblies of isolate 201A, visualised using Bandage.*

Each coloured section represents a contig from the assembly. A) Long read Oxford Nanopore Technology sequencing data of isolate 201A, assembled using Flye. It contains one contig with a length of 1,622,717bp. B) Short read Illumina sequencing data of isolate 201A, assembled using Shovill. the Illumina assembly contained 44 contigs over 100bp in size, and a total aligned length of 1,321,569bp. The contigs ranged in size from 150bp to 345,182bp in length.

5.3.6 Genome Assembly

All of the ONT sequencing data was then assembled using Flye. Ten of the thirteen assemblies resulted in a single contig that covered the length of the assembly, however, three of the samples contained more than one contig. These were 642A (3 contigs), 642B (5 contigs), and 286A (5 contigs). In the case of isolates 642A and 642B, the longest contig represented over 98% of the total assembly size. In 642A, one of the small contigs appears to circularise on the Bandage, and in 642B, all four of the smaller contigs seem to connect to each other with some overlapping sequence homology, although with some overlapping contig ends (**Figure 35**). This may indicate the presence of a plasmid in isolates 642A and 642B. In the assembly for isolate 286A, all of the contigs join together in one chromosome, but with some ambiguous sequence paths, possibly caused by repetitive sequence regions that are difficult to resolve.

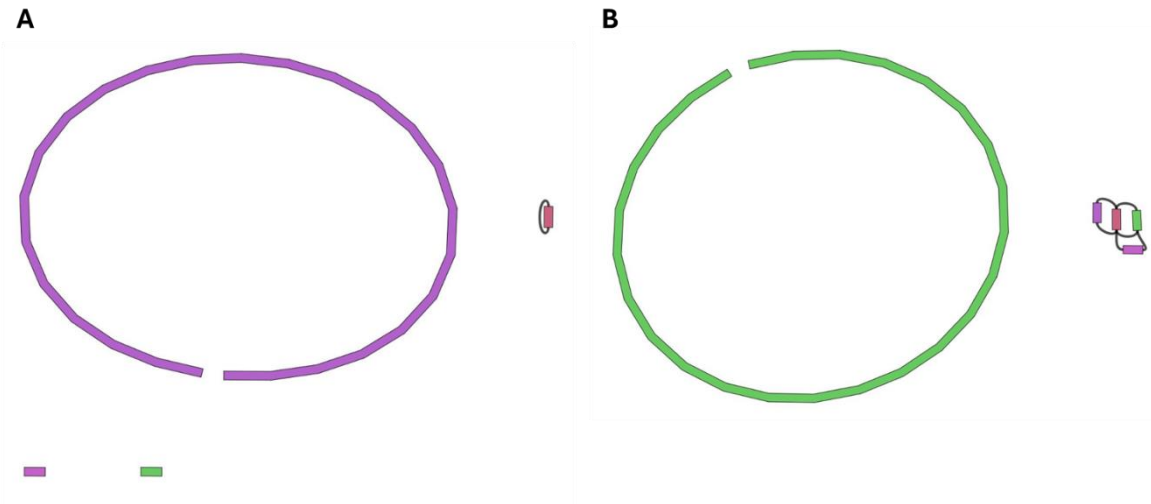


Figure 35. *Bandage plots of the Oxford Nanopore Technologies long-read Flye genome assemblies for two paired H. pylori isolates.*

A) H. pylori isolate 642A with one large circularised contig (purple) and 3 smaller contigs, one of which is circularized, possibly indicating the presence of a plasmid. B) H. pylori isolate 642B with one large circularised contig (green) and four short contigs that have overlapping end sequences, possibly indicating the presence of a plasmid.

5.3.7 Genome Polishing

The assembled ONT genomes were polished using the long- and short-read data. In all cases except for isolate 286A, the total length and the N50 value both increased. For isolate 286A, the total length decreased by 3155bp, however, the length of the longest contig increased by 4521bp (this isolate's sequence assembly contained 5 contigs). The number of contigs remained the same for all assemblies except for isolate 642B, which had one fewer contigs after polishing. The longest contig length and N50 value increased on average by 7112.62bp, while the total assembly length on average increased by 5922.62bp in length. The GC%

changed only by a negligible amount, increasing by 0.13% (**Table 23**).

Table 23. *QUAST assembly analysis results of sequences before and after polishing.*

Isolate		No. of Contigs	Largest Contig	Total Length	N50	GC (%)
256B	Flye Assembly	1	1540621	1540621	1540621	39.14
	After Polishing	1	1547569	1547569	1547569	39.15
	Difference	0	6948	6948	6948	0.01
875A	Flye Assembly	1	1654813	1654813	1654813	38.63
	After Polishing	1	1662109	1662109	1662109	38.74
	Difference	0	7296	7296	7296	0.11
935B	Flye Assembly	1	1654331	1654331	1654331	38.66
	After Polishing	1	1660477	1660477	1660477	38.85
	Difference	0	6146	6146	6146	0.19
201A	Flye Assembly	1	1622717	1622717	1622717	38.84
	After Polishing	1	1629810	1629810	1629810	38.96
	Difference	0	7093	7093	7093	0.12
11637	Flye Assembly	1	1673845	1673845	1673845	38.8
	After Polishing	1	1680895	1680895	1680895	38.83
	Difference	0	7050	7050	7050	0.03
642A	Flye Assembly	3	1562715	1581713	1562715	38.73
	After Polishing	3	1571273	1588051	1571273	39.04
	Difference	0	8558	6338	8558	0.31
733B	Flye Assembly	1	1623700	1623700	1623700	38.84
	After Polishing	1	1631740	1631740	1631740	38.89
	Difference	0	8040	8040	8040	0.05
201B	Flye Assembly	1	1617176	1617176	1617176	38.87
	After Polishing	1	1623282	1623282	1623282	38.99
	Difference	0	6106	6106	6106	0.12
286A	Flye Assembly	5	1145150	1627326	1145150	38.97
	After Polishing	5	1149671	1624171	1149671	39.06
	Difference	0	4521	-3155	4521	0.09
642B	Flye Assembly	5	1562536	1581177	1562536	38.72
	After Polishing	4	1570508	1583575	1570508	39.05
	Difference	-1	7972	2398	7972	0.33
733A	Flye Assembly	1	1624205	1624205	1624205	38.84
	After Polishing	1	1631698	1631698	1631698	38.89
	Difference	0	7493	7493	7493	0.05
875B	Flye Assembly	1	1659101	1659101	1659101	38.63
	After Polishing	1	1666148	1666148	1666148	38.74
	Difference	0	7047	7047	7047	0.11
934A	Flye Assembly	1	1626950	1626950	1626950	38.8
	After Polishing	1	1635144	1635144	1635144	38.93
	Difference	0	8194	8194	8194	0.13
Mean Difference		-0.08	7112.62	5922.62	7112.62	0.13

Initial long read assembly was performed using Flye, and then several rounds of polishing using long and short read sequencing data was performed using Racon and Medaka.

The genome sequence of the downloaded *H. pylori* NCTC 11637 (NCBI Accession number LS483488) reference strain (referred to as 'Reference') was used to identify changes in sequence content between the Nottingham *H. pylori* 11637 genome from before polishing ('Unpolished') and after several rounds of polishing ('Polished'). This was done as repeated passaging of the laboratory strain 11637 used at the University of Nottingham can cause the introduction of rearrangements and mutations. Pairwise NCBI BLAST alignments were performed between the Reference and Unpolished genomes, the Reference and Polished genomes, and the Polished and Unpolished genomes (**Table 24**). The BLAST percentage Identity score between the Reference/Polished genomes was higher than with the Reference/Unpolished. The Polished genome also had fewer gaps compared to the Reference genome than the Unpolished genome (62 vs 7190 respectively), and a higher number of sequence matches. The bit-score of the Polished genome was higher than the Unpolished when compared to the Reference genome, meaning that the sequence similarity was higher. The highest genome similarity bit-score was between the Polished and Unpolished data, although there was the highest number of sequence gaps between these two genomes (8027).

Table 24. *NCBI BLAST alignment comparison of three H. pylori 11637 genome assemblies.*

H. pylori 11637 Genome Type	Genome Size
Reference	1680937
Unpolished	1673845
Polished	1680895

Query/Subject	Identity Score (%)	Score	Identities	Gaps
Reference/Unpolished	99.41%	2.700e+06 bits (1462237)	1483538/1492391 (99%)	7190/1492391 (0%)
Reference/Polished	99.99%	2.755e+06 bits (1491685)	1491886/1491971 (99%)	62/1491971 (0%)
Polished/Unpolished	99.41%	3.043e+06 bits (1647731)	1671493/1681367 (99%)	8027/1681367 (0%)

'Percentage identity score' indicates the percentage of nucleotides that are the same between the two compared samples. The 'Score' is a log2 scale score of sequence homology likelihood (i.e. the sequence database size required to find a match to the query sequence by random chance) and can be used as a measure of sequence similarity. 'Identities' and 'gaps' columns indicate the number of nucleotides that have a correct match or a gap between the sequences, respectively. The 'Reference' genome refers to the H. pylori laboratory strain 11637 genome downloaded from NCBI (Accession number LS483488).

5.3.8 Resistance Genes Detected by the Comprehensive Antibiotic Resistance Database

All of the polished ONT genome assemblies were uploaded to the CARD online portal, and the Resistance Gene Identifier (RGI) tool was used. This tool compared the sequences to a curated reference library of antimicrobial resistance genes, and any identified sequence similarities were classified as 'Perfect', 'Strict' or 'Loose', whereby a 'Perfect' classification means that all the sequence amino acids correspond exactly to a reference resistance gene, and a 'Strict' and 'Loose' classification means that the sequence matches to a resistance gene with a bit-score of more than or less than 500 respectively (bit-score refers to a log scaled measure of

sequence similarity likelihood). All Identified resistance genes in the *H. pylori* sequences were categorised as 'Strict', with no 'Perfect' matches identified. All 13 genomes were identified as containing resistance mutations to 3 antibiotics; clarithromycin (23S rRNA), amoxicillin (*pbp2*), and glycopeptides such as vancomycin (*vanT*). Mutations in the *gyrA* gene that confer antibiotic resistance to fluoroquinolones were identified in 6 out of 13 sequences, *rdxA* and *frxA* mutations (conferring metronidazole resistance) in 8 out of 13 sequences, and *rpoB* (conferring rifampicin resistance) in 8 out of 13 sequences. No tetracycline resistance genes were identified by CARD. Sequence percentage identity of matching regions was over 96% for all identified resistance mutations, ranging from 96.19% to 99.54% sequence homology to genes in the CARD database, except for the *vanT* hits which were all 32-34% sequence identity.

The phenotypic resistance data for clarithromycin, amoxicillin, tetracycline, levofloxacin, and metronidazole were compared to the resistance genes identified from the CARD database for all the isolates, to identify if there was any accordance between the two sets of data (

Table 25). This comparison revealed that the identified resistance genes related to phenotypic resistance in differing amounts depending on the antibiotic. All tetracycline comparisons were in accordance, as no resistance genes had been detected, and no phenotypic resistance had been identified. For levofloxacin resistance, the CARD genes detected matched exactly with both the phenotypic resistance data, and the *gyrA* PCR data shown in Chapter 4. The amino acid substitutions identified by whole genome sequencing matched both the number and the type of substitution found using the Sanger sequencing, i.e. N87K, D91Y and D91G.

For clarithromycin, resistance mutations were identified in every sequenced sample, despite only 3/13 of the isolates showing phenotypic resistance. However, several different SNPs were detected within the 23S rRNA genes, and the 3 resistant isolates all contained the A2147G mutation, while none of the susceptible isolates did. All 13 of the sequences contained the 23S rRNA 1707T and A2144G substitutions which were identified by CARD as resistance SNPs, despite susceptibility status. All sequences showed the S494H and E572G resistance substitutions in the *pbp2* gene, although all isolates were phenotypically susceptible. Metronidazole had two genes identified that conferred resistance, *rdxA* and *frxA*, both of which had several possible resistance SNPs

available. The R90K substitution in the *rdxA* gene was present in all metronidazole-resistant isolates except 934A, which had a resistance phenotype but did not contain that mutation. Five isolates had a resistance phenotype without any identified metronidazole mutations, and isolate 935B had the resistance substitutions T31E, C49T and D59N detected despite susceptibility status. No glycopeptide phenotypic resistance data had been acquired, so it was not possible to compare the presence of the *vanT* in *vanG* gene cluster to the susceptibility testing results.

Table 25. *A comparison of resistance genes identified by CARD (The Comprehensive Antibiotic Resistance Database) and phenotypic resistance data acquired by E-test strip.*

		Clarithromycin	Amoxicillin	Levofloxacin	Tetracycline	Metronidazole
256B	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	***rdxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	T31E, R90K, C49T, D59N, H97Y
875A	Resistance Phenotype	S	S	R	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	***gyrA***	_	_
	Detected Mutation	C1707T, A2144G	S494H, E572G	N87K	_	_
935B	Resistance Phenotype	R	S	R	S	S
	Resistance Gene	**23S rRNA**	***pbp2***	***gyrA***	_	***rdxA***
	Detected Mutation	A2147G, C1707T, A2144G	S494H, E572G	D91Y	_	T31E, C49T, D59N
201A	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	**rdxA; frxA**
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	R16H, T31E, R90K, P106S, C49T, D59N ; A15V, Y62D
11637	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	_
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	_
642A	Resistance Phenotype	R	S	R	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	***gyrA***	_	_
	Detected Mutation	A2147G, C1707T, A2144G	S494H, E572G	N87K	_	_
733B	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	***rdxA; frxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	T31E, R90K, C49T, D59N; A85V, Y62D
201B	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	***rdxA, frxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	T31E, R90K, P106S, C49T, D59N; A15V, Y62D
286A	Resistance Phenotype	S	S	R	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	**gyrA**	_	***rdxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	D91G	_	R90K, H97T, C49T, D59N
642B	Resistance Phenotype	R	S	R	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	***gyrA***	_	_
	Detected Mutation	A2147G, C1707T, A2144G	S494H, E572G	N87K	_	_
733A	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	***rdxA; frxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	T31E, R90K, C49T, D59N; A85V, Y62D
875B	Resistance Phenotype	S	S	R	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	***gyrA***	_	_
	Detected Mutation	C1707T, A2144G	S494H, E572G	N87K	_	_
934A	Resistance Phenotype	S	S	S	S	R
	Resistance Gene	**23S rRNA**	***pbp2***	_	_	***rdxA; frxA***
	Detected Mutation	C1707T, A2144G	S494H, E572G	_	_	T31E, P106S, A67V, C49T, D59N; V7I, Y62D

Boxes highlighted in grey indicate a discord between the phenotypic resistance and genotypic resistance, and white boxes show agreement. Green boxes indicate phenotypic susceptibility to the relevant antibiotic, while red boxes indicate phenotypic resistance. The CARD resistance gene of interest is indicated in bold, with the associated resistance SNPs shown in the cell below. Dashes indicate where no resistance genes were identified by CARD.

When compared, the phenotypic and CARD genotypic antibiotic resistance status data, the results for amoxicillin and clarithromycin were highly discordant at 0/13 (0%) and 3/13 (23%) agreement respectively. Metronidazole had an agreement of 8/13 (61%), while levofloxacin and tetracycline both had a 13/13 (100%) agreement rate (**Figure 36**).

The sequencing data for the *H. pylori* NCTC 11637 isolate cultured and sequenced in Nottingham was compared to the NCTC 11637 reference genome data acquired from Genbank (accession number LS483488) on the CARD database to check if any resistance genes had been acquired during the repeated passaging of the bacteria during normal laboratory use. The genome sequence data from isolates 93A and 93B, acquired from sequencing work previously performed by Dr. Daniel Whiley, were also compared due to having different resistance profiles for levofloxacin. For the 11637 genomes, the CARD data comparison showed no difference in identified resistance genes but showed slight differences in sequence identity percentage for the *pbp2* resistance genes (98.02% and 97.54% for Nottingham 11637 and Reference 11637 respectively). The 98A and 98B genomes also had the same detected resistance genes but showed a >1% difference in gene identity percentage between the *rpoB* and *rdxA* CARD entries.

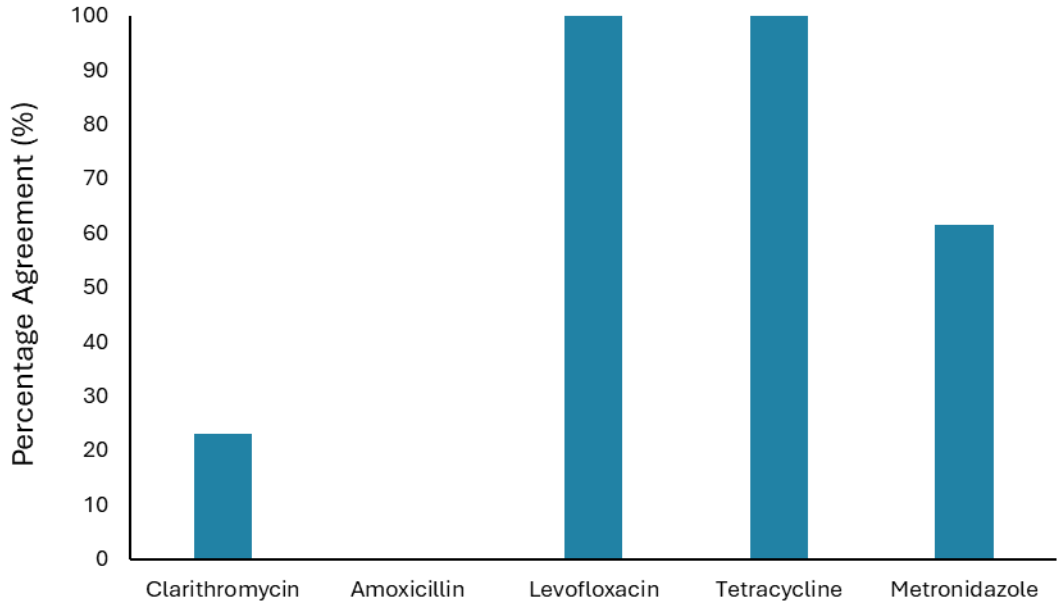


***Figure 36**. The percentage agreement between phenotypic resistance and CARD resistance genotype detection, by antibiotic.*

The agreement rate is the percentage of isolates where the phenotypic resistance data (acquired by culture-based methods) and genotypic resistance data (acquired by comparing whole genome sequencing data to the CARD database) were in agreement. 13 sets of resistance data were compared for each antibiotic.

5.3.9 Genome Annotation and Pangenome Analysis

The annotated polished ONT genome assemblies, as well as the previously acquired 93A and 93B sequences, were used to create a core genome alignment in Roary. The core genome is classed as genes that are common to all the sequences within the selection, shell genes are those that occur in 15-95% of sequences, and cloud genes are found in less than 15% of sequences. The total genes identified numbered 2753, however incomplete genes or

sequences that were too small were removed during core genome construction. Core genes made up 41.7% of the total, shells genes made up 717 (26%) of genes, and cloud genes made up 889 (32.3%) of the total number of genes (**Table 26**).

Table 26. *Roary pangenome subset ranges.*

Gene subset	Gene subset % range	No. genes within subset (%)
Core genes	(99% <= strains <= 100%)	1147 (41.7)
Soft core genes	(95% <= strains < 99%)	0 (0)
Shell genes	(15% <= strains < 95%)	717 (26.0)
Cloud genes	(0% <= strains < 15%)	889 (32.3)
Total genes	(0% <= strains <= 100%)	2753

The Roary gene presence and absence dataset was used to try and identify any genes or mutations present within levofloxacin resistant isolates. Although not a comprehensive analysis of the dataset, a cursory inspection revealed that some of the genes had been separated by Roary into two different 'groups'. Roary attempts to detect genetic paralogs (genes that are the result of a duplication event), based on the surrounding genetic sequences on either side of the gene of interest. Paralogs of the same gene are separated into different clusters and appear separately in the gene presence and absence file. In the case of the virulence gene *cagA,* three separate paralog groups of *cagA* were identified. Of the 14 sequences, 9 sequences contained *cagA* genes in Group 1, Group 2 contained *cagA* genes from 642A and 642B, and Group 3 only

contained *cagA* from sequence 935B. The two sequences from isolates 256B and 286A did not contain any *cagA* genes according to the Roary output.

The phylogenetic tree was constructed using a maximum likelihood model. The 'Polished' Nottingham laboratory NCTC 11637 and the downloaded 'Reference' NCTC 11637 genome strains were very closely related to each other but grouped separately somewhat from the rest of the tree. The paired isolates from the same patients had very high levels of similarity, with isolates 642A and 642B being grouped slightly apart from the rest. The phylogenetic tree did not seem to show a link between resistance and relatedness aside from in isolates taken from the same patient. No relationship could be ascertained between phenotypic resistance and accessory gene presence or absence **Figure 37**.

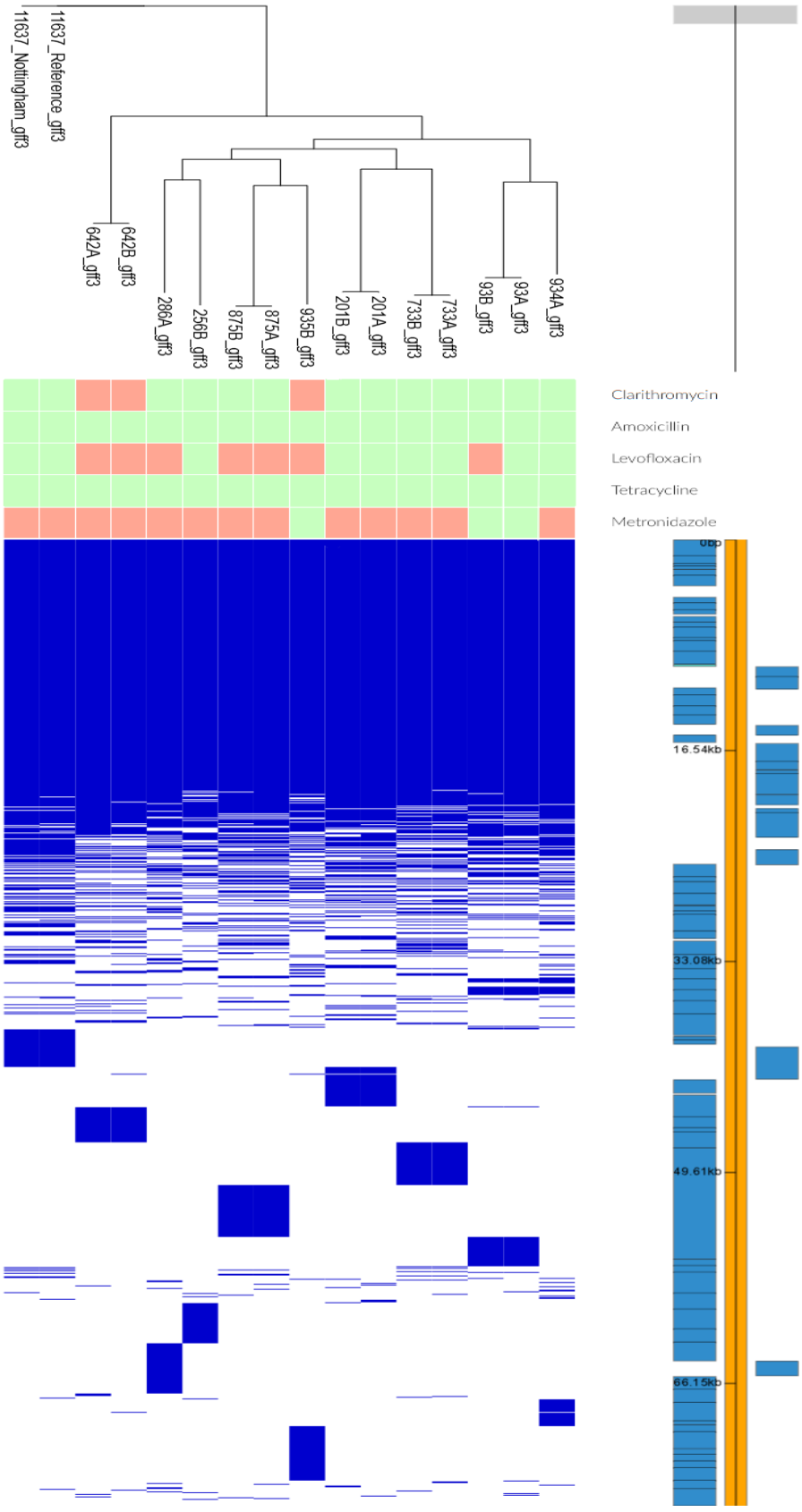


Figure 37 *- Phylogenetic tree and core genome alignment of H. pylori genome sequences, including antibiotic resistance metadata.*

Green boxes indicate susceptibility to the corresponding antibiotic, and red boxes indicate resistance. Dark blue sections indicate the presence of a gene, with the solid blue section indicative of the core genome. The orange and light blue bars on the right hand indicate a size scale for the genome and associated genes. The phylogenetic tree was created using RAxML, which generates a maximum likelihood phylogram. Image made using Phandango.

5.4 Discussion

5.4.1 Main Findings

The results of this chapter demonstrate that polishing can be performed on ONT genome assemblies to improve quality and enable SNP detection. Polished ONT genomes can be used with online tools such as CARD to identify resistance genes, with 100% agreement shown between detected levofloxacin resistance genes and resistance phenotype. Additionally, sequences assembled from long-read data can provide insight into the location of genes within a chromosome, which can have impacts on gene expression.

5.4.2 Long and Short Read Genome Assembly

In this study, we show that ONT sequencing can be used to create a complete genome of *H. pylori* with gene locations within the chromosome preserved. Despite the less accurate per-base sequencing quality in comparison to Illumina sequencing, the context of gene location can provide important information that is obscured in assemblies with dozens of contigs.

The assemblies created from the Illumina data were extremely fragmented, with the 201A assembly containing at least 44 contigs. This is likely due to areas of highly repetitive DNA sequences and duplicated genes, which do not provide enough information with short-read data to properly resolve into the correct order during assembly. This is a known drawback of *H. pylori* short-read sequencing [349], and can interfere with the identification of highly homologous yet distinct gene regions. Previous studies have shown that this can interfere with investigations of *H. pylori* virulence factors. The outer membrane protein BabA is responsible for binding to host cell blood group antigens, which is an important step in colonisation. However, the genes *babA*, *babB* and *babC* are highly similar and can undergo homologous recombination to produce different variations of the BabA protein. The variants can bind to a wider range of host cell blood group proteins, aiding infection establishment. It was shown that Illumina sequencing resulted in contigs that terminated at the *bab* genes and truncated the sequences, masking important data [411]. In comparison, n early every ONT assembly produced a complete, circularised genome without any junctions at contig edges. This allows for a clearer characterisation of the genome. In the future, additional assembly methods such as hybridSPAdes [412] could be investigated for comparison.

5.4.3 Polishing of Long-Read *H. pylori A*ssemblies Improved Sequence Accuracy

In this study, it was shown that polishing an ONT long-read assembly using long- and short read data could increase the identity score, decrease the number of genome gaps, and decrease the size difference between a *de novo* assembly and a reference genome of the same strain.

On average, the Q score for the ONT data used in this study was 11.2, indicating a 0.079 probability of an incorrect base call (or 92.057% base call accuracy), which is within the expected range for Oxford Nanopore sequencing data. However, ONT read quality can decrease further when base-calling a region of repeats of the same nucleotide [362], which can lead to gaps and inaccuracies within the sequence. Genome polishing was performed to assess if the quality of the assembly could be improved.

NCBI BLAST was used to compare the 'Reference' 11637 sequence with the Nottingham 11637 sequence from before and after polishing. The alignment of the 'Unpolished' Nottingham 11637 sequence identity was 99.41%, while the 'Polished' 11637 genome had a 99.99% percentage identity. Although a 0.58% sequence identity mismatch appears to be a trivial amount, in a 1.6 million bp sequence, 0.58% amounts to around 9700 bases, and when

searching for SNPs any of those sequencing errors may cause a false positive result.

It is to be expected that the highest similarity was between the polished and unpolished sequences, however, they also had the highest number of gaps between them (8027). The introduction of sequence gaps may have been a result of sequencing errors during regions of repeated bases [413]. Before polishing, the ONT assembly was shorter than the reference sequence by 7072bp, while after polishing, the polished genome was only shorter than the reference genome by 42 bases.

Care must be taken not to over-polish a genome, which may result in errors from the short-read sequences being incorporated into the assembly sequence [383], [414]. Ensuring that short-read sequencing data is high quality is essential to the polishing process. In this study, the quality of the Illumina sequencing data was closely assessed beforehand to ensure it would be suitable for polishing. The polished 11637 genome was closer in size and BLAST percentage identity to the reference 11637 genome than the unpolished sequence was, suggesting that the polishing process successfully improved the genome completeness and accuracy. Isolate 642B also had one contig less after polishing, as it may have been incorporated into one of the other contigs during the polishing process.

The polishing process itself can be largely automated and streamlined using Galaxy workflows (a feature that allows a sequence of tools to be linked together into one operation).

An alternative to performing multiple rounds of polishing on an initial long read assembly would be a hybrid assembly approach such as hybridSPAdes [412] or Unicycler [415], which are all-in-one pipelines that use both long and short reads together to quickly produce an assembly. This has the advantage of being quicker without requiring multiple rounds of polishing. However, in the case of hybridSPAdes, it takes a short read assembly approach first, which in the case of highly mutable bacteria like *H. pylori* [416] would still experience the same assembly issues caused by a highly plastic genome structure. A comparison spanning more assembly types would be a useful future project. Additionally, this project would have benefited from a more in-depth look at the qualitative changes of the sequences before and after polishing such as those performed in Lee *et al*., (2021) [383], which compared several different types of polishing tools, however time constraints prohibited this.

Currently, the read accuracy of ONT sequencing data of up to 99% is possible when using the latest Q20+ flow cells and super high accuracy base-calling models [417]. However, the Q20+ flow cells are not widely used yet. With further development and

dissemination, however, ONT data in the future may become of a high enough quality that polishing is not required. Polishing of ONT genomes is a highly useful process for producing high-quality genomic data, and based on this research seems to be effective for creating whole genome sequences that can be used for SNP detection in resistance genes. However, genome polishing is currently too expensive and time-consuming for widespread diagnostic use for *H. pylori* treatment in clinical settings. For now, its utility is based in research, and for detecting and identifying interactions between genes that can increase resistance. The information gleaned from that research can then be applied in a clinical setting, using cheaper and quicker methodologies such as PCR. Until ONT sequencing itself is of a high enough quality to compete with Sanger sequencing for SNP detection, it is unlikely to find widespread adoption for AMR detection within clinics in the near future.

5.4.4 The CARD Tool can Successfully Identify Levofloxacin Resistance Genes in *H. pylori*.

The CARD Resistance Gene Identifier tool successfully identified SNPs linked to levofloxacin resistance with 100% agreement to the phenotypic resistance data. The *gyrA* amino acid substitutions N87K, D91Y, and D91G that are linked with levofloxacin resistance [199] were identified in the phenotypically resistant isolates, while

no resistance genes were detected in the phenotypically susceptible isolates. The number and type of *gyrA* amino acid substitutions detected by CARD in the polished ONT genomes were the same as those found in the Sanger sequencing data for these isolates, and the agreement between genotypic and phenotypic resistance was 100%. This is a promising outcome for the detection of levofloxacin resistance using genomic sequencing data.

The metronidazole CARD data had the biggest range of possible resistance amino acid substitutions, in both the *rdxA* and *frxA* genes. Metronidazole acts by causing DNA breakages but is only functional when activated by the bacterial NADPH nitroreductase enzyme. Therefore, the mutation of the *rdxA* gene that encodes the activating nitroreductase can lead to metronidazole resistance [418]. The mutations in *rdxA* only need to reduce the RdxA protein's reductase activity. *H. pylori* can grow and survive without a functional *rdxA* gene, and as such there is a large array of mutations seen in this dataset that can serve the purpose of inactivating the gene [419]. The mutations in the *frxA* gene are associated with increased metronidazole resistance when present with *rdxA* resistance mutations but do not usually confer resistance themselves [420]. The R90K substitution was present in all except one of the resistant isolates and is closely linked with metronidazole resistance [421]. Unlike with the other antibiotics,

however, there were five cases of metronidazole-resistant isolates with no mutations flagged by CARD. This implies that there are other metronidazole resistance mutations that either have not been discovered yet or are not currently present in the CARD database.

Concerning clarithromycin, the CARD database returned a total of three mutations in the 23S rRNA gene that it associated with resistance – the SNPs A2147G, C1707T, and A2144G. CARD does not show any indication about which copy of the two 23S rRNA genes the mutations in each genome are found on. In all sequences, the 23S rRNA mutations C1707T and A2144G were identified by CARD as being present. However, these mutations appeared regardless of if the related isolate was phenotypically resistant or susceptible to clarithromycin. The mutation A2147G was only present in the three clarithromycin-resistant isolates in the set, and not present in any of the susceptible isolates, which strongly suggests that it is related to resistance. Previous reports have also shown that the 23S rRNA A2147G mutation is linked to high levels of clarithromycin resistance [316], [422], which corroborates the findings shown in this study.

Two clarithromycin resistance mutations were identified by CARD as being present in all isolates, however, their relationship to phenotypic resistance seems more ambiguous. Only two papers that mentioned the C1707T mutation in the 23S rRNA gene for *H.*

pylori could be identified. In both papers, it was noted that C1707T was a novel mutation in the localities of the papers (China [408] and Mongolia [423]). However, the paper from China by Fang *et al*., (2024) had used the CARD database to identify the C1707T mutation within their dataset and noted that all isolates within their study contained that mutation, which consisted of 37 clarithromycin-resistant and 11 clarithromycin-susceptible isolates. That paper also expressed uncertainty as to whether C1707T is linked to clarithromycin resistance [408]. When consulting the initial paper from Mongolia that linked the C1707T mutation to clarithromycin resistance, it appears that the C1707T mutation was only found in a single clarithromycin-resistant isolate, which casts doubt on its practicality for identifying clarithromycin resistance [423].

When considering the third clarithromycin SNP identified by CARD, previous studies have found that the A2144G mutation is strongly related to clarithromycin resistance [424], [425], while others have found the mutation in both resistant and susceptible isolates, like in the data in this paper [408]. This may indicate that the A2144G mutation provides a low level of protection to the antibiotic, possibly due to a lower clarithromycin MIC associated with this SNP [426]. This makes predicting phenotypic resistance using this mutation more difficult.

When looking at amoxicillin resistance, the same two amino acid substitutions in the *pbp2* (penicillin-binding protein) gene were found in all isolates – S494H and E572G. The agreement rate between amoxicillin phenotypic and genotypic resistance was 0% for amoxicillin, however, none of the isolates in this dataset were amoxicillin resistant, making comparison harder. The S494H and E572G amino acid substitutions have not been shown to confer amoxicillin resistance on their own but may act to increase the amoxicillin MIC when found in combination with resistance SNPs in *pbp1* and *pbp3* [193]. However, little other data on these substitutions in *H. pylori* could be found. No isolates with tetracycline resistance have been found in the Nottingham Strain Collection, and no tetracycline resistance genes were identified by CARD in the sequencing dataset.

Aside from the first- and second-line antibiotics commonly used in the treatment of *H. pylori*, CARD also identified genes conferring resistance to glycopeptides. All sequences were identified as containing the *vanT* gene in *vanG* cluster that confers resistance to antibiotics such as vancomycin, however, in this study, no phenotypic data on glycopeptide resistance has been collected. Glycopeptides are not used in the treatment of *H. pylori* infections in the UK, as Gram-negative bacteria are naturally resistant to this type of antibiotic [427]. The homology of the *vanT* genes within the sequences was low (~32% for all sequences), however, it was

still classified as 'strict' i.e. with a bit-score sequence similarity likelihood of more than 500. Only one paper was found that mentions *vanT* genes in *H. pylori*, and in that case the genes were again identified by the CARD database [428]. The authors of that paper checked for *vanT* in a Prokka annotation of the same genome, which also showed a *vanT* annotation.

Consulting the database used by the Resistance Gene Identifier, the *vanT* gene in question encodes a membrane protein from the bacteria *Enterococcus gallinarum* [429]. Although there may be some homology to the *E. gallinarum vanT* gene found in the genome sequences, the glycopeptide resistance in *H. pylori* is most likely due to the lipopolysaccharide layer that surrounds the peptidoglycan target site, as is the case with the majority of Gram-negative bacteria [430]. The Roary presence/absence table created using the annotated ONT genome sequences were searched, however, the *vanT* gene was not identified as present among the annotated sequences.

Based on the comparisons to phenotypic data shown in this study, the CARD database performed well in identifying the presence of levofloxacin resistance genes. This concurs with previous findings which showed complete agreement for tetracycline and ciprofloxacin CARD genes identified, and phenotypic resistance in *Campylobacter jejuni* [431]. Similarly, the same paper described

that beta-lactamase genes were identified in 2 of the isolates while the corresponding protein product and phenotypic resistance profiles were not detected [431], which is similar to the results shown in this chapter that highlighted amoxicillin resistance genes present in isolates that did not display phenotypic resistance.

The resistance genes detected for clarithromycin and amoxicillin provided valuable information for the prediction of antibiotic resistance, however, in the cases of metronidazole and clarithromycin, the output was less clear. Some genes are consistently associated with resistance while others act more to modulate antibiotic MIC in the presence of other genes. This information is not distinguished by the CARD output, nor is there any indication of mutations that have very limited evidence linking them to resistance. Additionally, some resistance mechanisms may be missing from the database.

When compared to other resistance gene databases like NDARO (National Database of Antibiotic Resistance) [432] and PointFinder [433], the CARD database has a good level of *H. pylori* representation, with 1852 NCBI whole genome sequences represented in the Resistance Gene Identifier tool, as well as homology identification for thousands of other sequences. According to a review of the three databases in 2022 [434], The *Helicobacter* microbial group was not represented in the NDARO

database. While it was one of 10 pathogens included in the Pointfinder database, it was the least represented. CARD represented the largest number of microbial groups (37 genera), but *H. pylori* representation was comparatively low [434]. Another review [428] of *H. pylori* representation in resistance gene databases compared CARD to other resistance gene databases such as ARG-ANNOT (Antibiotic Resistance Gene – Annotation) [435], MEGARes [436] and ResFinder [437]. It was found that ResFam and ResFinder did not contain as many *H. pylori* specific AMR genes compared to the CARD and MEGARes databases. Additionally, the authors of that paper found that curation of the gene output is necessary, and that all the different databases will produce different results based on database contents as well as detection methodology, such as amino acid sequence homology detection vs BLAST alignment methods. Based on the findings in this chapter and in agreement with previous literature, CARD provides good representation of *H. pylori* resistance data, but care is required when interpreting results. These services are reliant upon the collaborative effort of researchers to discover resistance genes, and curators to expand the databases. While this is a labour-intensive process, tools are being developed to help streamline database curation. A paper released in 2023 demonstrates a tool that highlights which new publications contributors should prioritise for addition to the database [438].

The problem faced with CARD is that antibiotic resistance is based on a multitude of factors, the nuances of which can be hard to communicate while also keeping CARD accessible and easily understandable to a wide range of users. As with many open-source databases, it requires a lot of work to curate and maintain the information. Despite some inaccuracies, it is still a useful tool which can be used to quickly highlight mutations within a sequence, with the caveat that care and knowledge is required by the end user to double-check the outputs to interpret what the information means.

5.4.5 Roary Paralog splitting

The Roary gene presence and absence data in this chapter showed that the *cagA* gene was not present in all genomes. In the ones that it was present in, Roary had split the genes into different groups, indicating that there were possible paralogs present. The Roary pangenome tool features a mechanism for splitting gene paralogs using information from the surrounding 'gene neighbourhood' to group them into appropriate categories [405]. That is, the context of the surrounding gene sequence is considered when deciding whether a similar gene in two different sequences is the same. Homologous genes are two separate yet comparable genes and are defined by high levels of sequence similarity due to originating from the same shared ancestral gene.

There are two reasons that homologous genes may occur, the first being speciation, in which case the two genes are 'orthologs' of each other. The second reason is that if a gene within a bacterium becomes duplicated the resultant pair of genes are called 'paralogs' of each other [439]. *H. pylori* isolates have been identified that contain multiple copies of *cagA* [440] which can have consequences for bacterial pathogenesis due to *cagA* being significantly associated with the development of gastric cancer [441].

Due to the highly mutable nature of *H. pylori*, it could also be the case that the three groups of the *cagA* 'paralogs' may instead indicate where the *cag* pathogenicity island containing the *cagA* gene translocated to different areas on the chromosome, and the different 'gene neighbourhood' reflects this. The position in the chromosome and the orientation of the gene after translocation might have had an impact on transcription and expression. Increased distance from the origin of replication (*oriC*) in a bacterial chromosome can decrease gene expression, as can distance from the relevant transcription factor [442].

When a gene translocates it can also re-integrate into the chromosome in the middle of a different gene, causing the split gene to express incorrectly or become non-functional [443]. This further enforces the importance of long-read sequencing data for

the determination of genome evaluation, as short-read data would obscure this information. Roary has previously been used with *H. pylori* genomes to identify gene recombination events, particularly in regard to phage populations [444]. Additionally, the different Roary groups of *cagA* might indicate genetic differences in the *cagA* gene itself, for instance the EPIYA motifs that vary in Western-type and East-Asian-type CagA proteins [445], as discussed in section 1.5.4 (EPIYA Motifs and Virulence). Further analysis such as a BLAST comparison of the *cagA* genes in the WGS data would be required to identify if this is the case, however time constraints on this project prevented this analysis from occurring.

Paralogous groups of *vacA* were also identified within the dataset, however the *hom* gene was present as a core gene across all sequences and was not separated into different groups, unlike in previous studies [446].

No *babA* genes were identified in the gene presence and absence data, which may be caused by a lack of representation of *H. pylori* data in the default data that Prokka references for genome annotation. BabA is a highly important protein in *H. pylori* adhesion during colonisation, and so it is expected that it would be present in the dataset. However, it is a *H. pylori* specific gene and a cursory search of two of the databases that Prokka uses to source gene data show that the Bacterial Antimicrobial Resistance Reference

Gene Database [447] does not contain any reference to *H. pylori*, although there are five results from IS Finder, which is a database of bacterial insertion sequences [448]. It is also possible that the *babA* genes are present in the Roary output but represented by a different name originating from a similar gene from a different bacterial species. In the future, a curated *H. pylori* specific annotation should be used, such as was done in Wilkinson *et al*., (2023) [349], which would allow for more insight to be gained from the Roary output. The genome annotation in this chapter has room for improvement, and future work would address this.

The recombination rate in *H. pylori* is higher than in most other measured bacterial species [449], as there are fewer DNA mismatch repair mechanisms and additional strategies for importing sections of environmental DNA [416]. Therefore, gene translocation is a frequent occurrence. The phylogenetic tree created from the assembled *H. pylori* genome sequences doesn’t seem to show any distinct lineages between the *cagA* paralog groups, which may be because the gene translocation event occurred more than once, or a translocated gene was lost in subsequent generations. The high plasticity of the *H. pylori* genome makes these options plausible, although a more thorough understanding could be gained by including a wider range of *H. pylori* sequences when constructing the phylogenetic tree in the future.

5.4.6 Conclusion

As tools and technology develop, WGS will become more accessible and functional. The current problems facing those who wish to undertake WGS is acquiring enough processing power to complete data analysis, and understanding the technical aspects of bioinformatics. Machine learning for gene associations and for predicting protein function is constantly improving and developing, and databases of AMR genes will continue to be refined and updated. Collecting WGS data is also a useful investment, as provided that adequate data storage is available, it is a resource that can be used globally at any point in the future. Technological breakthroughs can be used retroactively on the data, providing valuable historical information. However, the cost and time of WGS compared to PCR-based SNP detection methods for clinical use is highly prohibitive for widespread use. WGS is currently more suitable for research into as yet unknown resistance mechanisms and gene interactions, while PCR and Sanger sequencing would be more accessible and cost-effective at a clinical level while still maintaining an acceptable level of diagnostic accuracy. Finally, the findings in this chapter indicate that WGS analysis can be aided by bioinformatics tools and online databases, however many of them do not have a comprehensive representation of *H. pylori*. Their usefulness may increase in the future as more data becomes available.

Chapter 6: Final Discussion

6.1 General Discussion

The initial focus of this thesis was to discover the scope of the problems presented by antibiotic resistance in *H. pylori*, and then identify what steps could be taken to address them. The gap in knowledge of local AMR rates can cause considerable negative consequences for treatment outcomes and patient well-being. With that in mind, the major aims of the research were to assess the antibiotic resistance profiles of the Nottingham Strain Collection, and to identify the mechanisms and relationship between phenotypic and genotypic antibiotic resistance to levofloxacin in *H. pylori*. Additionally, a further aim was chosen to assess the suitability of different sequencing techniques for identifying resistance SNPs. With antibiotic resistance in *H. pylori* increasing and spreading, we must know the scope of the problem to more effectively work towards remedying it. The results of this thesis have contributed to that solution.

6.2 Major Findings and Recommendations

As discussed in Chapter 3, there is little up-to-date research about the current state and scope of *H. pylori* antibiotic resistance in the UK. The work performed in this thesis addressed this knowledge gap by successfully characterising the antibiotic resistance profiles

of 241 *H. pylori* isolates in the Nottingham Strain Collection. Of particular interest were the results that revealed unexpectedly high levels of resistance to first-line antibiotics, particularly clarithromycin (27.8% resistance) and metronidazole (61.8% resistance). Overall, 67% of the isolates displayed resistance to at least one type of antibiotic, and multi-drug resistance levels were high with 27% resistant to 2 or more antibiotics. When considered in the context of European health guidelines, the clinical significance of these findings becomes apparent, as local clarithromycin resistance rates are higher than the recommended levels for effective eradication therapy meaning that alternative antibiotics should be selected where possible.

Unfortunately, without resistance data, clarithromycin is generally selected for therapy regimens, and treatment success rates suffer. Treatment failure is a known contributor to the development of antibiotic resistance, and this is enforced by the findings which show that patients who had undergone previous eradication therapy had significantly higher resistance rates to clarithromycin and metronidazole compared to those who did not have any record of previous antibiotic treatment. Resistance also significantly increased over time (2001-2005 to 2011-2018) up to 40% for clarithromycin and up to 78% for metronidazole. Based on this data, and in line with European guidelines, clarithromycin should not be used as a first-line treatment and alternatives should be

considered. This chapter represents the first major data on *H. pylori* resistance in the UK for over a decade, and the first in the Nottingham area.

Further evidence was presented that showed paired isolates from the antrum and corpus of the same patient with mismatches in the antibiotic resistance profile or the CagA/VacA virulence factor type, demonstrating that multiple populations might be living concurrently in the stomach, which has implications for testing and treatment.

In Chapter 4, the focus of the research then shifted to what the causes might be for so little resistance data being collected in the UK, and how this could be addressed. Despite UKHSA guidelines, culturing is rarely performed even after several failed rounds of eradication therapy, and antimicrobial susceptibility testing is a long and involved process. An alternative to culturing is PCR amplification and sequencing of resistance genes of interest, which was performed on the *gyrA* genes of 38 *H. pylori* isolates. Among the major findings of this chapter were that GyrA amino acid substitutions Asp91Gly, Asp91Tyr and Asn87Lys are closely associated with resistance to levofloxacin, and that genotypic levofloxacin resistance is largely consistent with phenotypic resistance.

Based on the data presented in this thesis, it can be recommended that PCR based detection methods could be employed to confidently screen for levofloxacin resistance in *H. pylori* isolates. The major resistance SNPs can be reliably detected and identified using this method, although there are still causes of resistance that may exist outside of the QRDR that are not currently recognised.

With that in mind, although resistance rates in the Nottingham Strain Collection were comparatively low, the importance of levofloxacin in the treatment of drug-resistant TB makes it unsuitable for widespread use in an unrestricted manner. It cannot be recommended that levofloxacin be extensively prescribed without resistance testing protocols in place. There are several papers from authors globally that identify levofloxacin resistance genes and compare them to resistance data [307], [311], [318], but as far as the author is aware this thesis is the first in 15 years to present data on which specific QRDR mutations are present in the UK [221], and compare them to phenotypic MIC data. It is important to continue monitoring which levofloxacin mutations are found in the UK within resistant isolates. *H. pylori* resistance mutations can vary based on locality [28], and knowing which mutations are disseminating in different locations can help combat the spread of resistance and ensure levofloxacin is being used in a responsible way.

In Chapter 5, further research was conducted on how phenotypic resistance can be influenced by genes outside of those that encode antibiotic target proteins, and how this could be detected using whole genome sequencing methods. The findings in this chapter showed that Illumina and Oxford Nanopore Technologies sequencing of *H. pylori* can be used to create complete *de novo* genome assemblies. Genome polishing can be employed to make the sequences high enough quality to use for SNP identification and the detection of AMR genes. This can be performed using the Resistance Gene Identifier tool in the CARD website, although manual curation of resistance results is advised.

This data is important in that there is little published data comparing *H. pylori* genomic sequencing data to CARD Resistance Gene Identifier genotypic output. Two papers have used CARD with *H. pylori* Illumina data to aid in identifying resistance genes [450], [451], however, this study is novel in exploring the relationship between the CARD RGI output and phenotypic resistance in *H. pylori*.

Based on this, it can be recommended that polished *H. pylori* WGS data can be used with confidence to identify resistance and virulence genes, although currently, the process is likely too time-consuming and expensive for clinical applications [452]. However, assembled genomes can be utilised in a research setting to

examine gene interactions and mechanisms that aid colonisation and immune evasion. The comparison of different types of sequencing assemblies is something that has been performed before with other types of bacteria, however there are very few bacteria that undergo as much gene movement and chromosomal rearrangement as *H. pylori [416]*. This has possible implications for resistance gene expression, and this chapter began to explore that connection. In the future, there is far more information that could be discovered on this subject.

6.3 Context of the Thesis Results

This study has created a solid foundation for calls to reexamine the way *H. pylori* antibiotic treatment is managed in the UK. Although *H. pylori* infection rates have been declining in many areas [260], this cannot become a cause for complacency towards monitoring and treatment. Increasing resistance rates and the spread of AMR genes globally have the potential to jeopardise the progress that has so far been achieved if left unchecked [453].

In 2016, the results of a survey on microbiology testing laboratories in the UK was published, showing that of 170 laboratories that participated in the audit, 22 performed *H. pylori* culturing from biopsies on site, and only 9 of those performed any kind of antibiotic susceptibility testing. Most of those processed fewer than one isolate per week, and resistance data was generally

not collected. Only 2 laboratories tested for levofloxacin resistance [454]. Little data is available to identify if testing rates have improved since then. While it is true that testing for *H. pylori* AMR costs time and money that is already very scarce, in the long run it has been shown in other countries that treatment failure and disease progression can become decidedly more expensive as patients repeatedly return and require hospitalization, particularly in cases of cancer development [153].

There are many avenues of discovery that show promise in improving the future of *H. pylori* resistance testing and treatment. PCR-based tests that utilize faecal matter to identify AMR genes are becoming more widely available [455], as well as the probe-infused paper strip-based HelicoDR [456] that can detect mutations in *gyrA* amino acid positions 87 and 91.

In terms of whole genome sequencing for more comprehensive AMR and virulence factor detection, one of the largest barriers remains the need for biopsy procedures. Techniques that avoid the need for biopsy are being investigated, however, such as capsules containing a sponge that can be swallowed. The sponge is connected to a string that can be used to retrieve it after a set time, after which point enough *H. pylori* bacteria have adhered to the sponge to possibly culture a sample from it [259]. Materials that selectively bind to *H. pylori* antigens and aid with attachment

may also prove useful. Currently, extracting DNA directly from biopsies without culturing and growing the bacteria is still difficult due to high levels of contamination from oral microflora and human DNA, but techniques to circumvent this problem may be discovered in time.

Following previous trends, whole genome sequencing is likely to become less expensive, more accurate, and more generally accessible in the future [457]. The vast quantities of data obtained in this way can be analysed using advances in machine learning that can identify previously unknown AMR gene interactions [458], and help with predicting how mutations might impact protein function and binding [459].

This study was started with the focus of helping to combat antimicrobial resistance, which is an urgent global crisis [460]. Antibiotic stewardship is of both scientific and ethical importance, and in that context; this study has contributed knowledge to that cause.

6.4 Evaluation of Experimental Design and Execution

The content and procedures used in this thesis developed over the course of several years, and in some cases, limitations in methodology have since become apparent.

One drawback to studying the Nottingham Strain Collection is the lack of access to some of the patient data. While a large amount of anonymised patient information was provided with informed consent, much of the patient history was unknown including what previous antibiotics had been prescribed both for *H. pylori* treatment and for other infections. This would provide valuable insight into the development of secondary antibiotic resistance. Information was provided about whether a patient had previously undergone eradication therapy, however the treatment specifics were unknown.

There were also some inherent biases introduced into the data. Firstly, only patients suffering from dyspepsia were referred for an endoscopy, and so the samples taken were not representative of the general population of those infected with *H. pylori*. Additionally, when isolating and culturing the bacteria, it is likely that some selection occurred that favoured those that grow well in laboratory conditions. This was further compounded when selecting isolates for sequencing, due to high amounts of growth needed for DNA extraction.

Financial factors were also a consideration. Initially, the cost of acquiring E-test strips was an obstacle, and so disc diffusion AMR testing was performed on many of the isolates of the Nottingham Strain Collection. However, later when E-test strips became

available, it meant that validation needed to be performed to ensure the two datasets could be used together. Additionally, MIC data is now lacking for isolates that were tested using disc diffusion, requiring further time and money to re-culture them and obtain these results. Whole genome sequencing is also an expensive process, and therefore the number of strains that could be included in that dataset was limited. Time constraints also limited the amount of analysis that could be performed on the WGS data.

A particular error in experimental design concerning rifampicin AMR testing was identified only after work had been completed. It had been previously thought that rifampicin antibiotic discs and E-test strips could be used to predict resistance to the third-line antibiotic rifabutin [164]. However, later research demonstrated a lack of cross-resistance [209] and therefore the results obtained were invalid, resulting in a loss of time and resources.

Equally, this thesis demonstrates a number of merits. The data presented in the thesis is novel and important information concerning antibiotic resistance data in the UK, which had previously been lacking. The MIC data that had been acquired for part of the Nottingham Strain Collection can be used for further work, particularly as some of the associations between virulence factors and resistance profile are yet to be analysed. Similarly, the

whole genome sequences that were assembled during this research are useful resources for future studies, with a multitude of analyses that can be performed. Despite the serious disease outcomes that can be caused by *H. pylori* infection, it is often underrepresented in terms of sequencing data and this thesis has gone some way to addressing that.

6.5 Future Work and Conclusions

The Nottingham Strain Collection will continue to be tested for AMR profiles, with all the isolates from previous years having full MIC data collected. Additionally, the research group has resumed work with clinicians from the Queens Medical Centre to obtain gastric biopsy samples and expand the isolate collection. This will be used to further monitor *H. pylori* AMR levels, and additional patient information means that further associations can be elucidated. The cultures from the biopsy samples will also be used in deep population sequencing studies that will aim to identify genetic variation within a population as opposed to single colony isolates.

A further aim is to obtain the whole genome sequencing data of all isolates within the collection, and from clinical strains originating from other geographic locations. This will drive research within the group that aims to use machine learning with WGS data to identify genes related to resistance and disease. Antibiotic resistance is a

multifaceted problem which will require continued progress and collaboration to overcome.

Bibliography

[1] K. Robinson and J. C. Atherton, ‘The Spectrum of Helicobacter-Mediated Diseases’, *https://doi.org/10.1146/annurev-pathol-032520-024949*, vol. 16, pp. 123–144, Jan. 2021, doi: 10.1146/ANNUREV-PATHOL-032520-024949.

[2] ‘World Health Organization - Cancer’. Accessed: Feb. 05, 2024. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/cancer

[3] ‘List of Classifications – IARC Monographs on the Identification of Carcinogenic Hazards to Humans’. Accessed: Dec. 31, 2024. [Online]. Available: https://monographs.iarc.who.int/list-of-classifications/

[4] ‘WHO publishes list of bacteria for which new antibiotics are urgently needed’. Accessed: Jan. 22, 2024. [Online]. Available: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

[5] McNulty C., ‘Test and treat for Helicobacter pylori (HP) in Dyspepsia: Quick Reference Guide for Primary Care’, 2017. Accessed: Jul. 29, 2020. [Online]. Available:

https://www.gov.uk/government/publications/helicobacter-pylori-diagnosis-and-treatment

[6] E. C. Rosenow and A. H. Sanford, 'The Bacteriology of Ulcer of the Stomach and Duodenum in Man', *J Infect Dis*, vol. 17, no. 1, pp. 219–226, 1915, Accessed: Dec. 27, 2023. [Online]. Available: https://www.jstor.org/stable/30083497?seq=5

[7] B. J. Marshall and J. R. Warren, 'Unidentified Curved Bacilli In The Stomach Of Patients With Gastritis and Peptic Ulceration', *The Lancet*, vol. 323, no. 8390, pp. 1311–1315, Jun. 1984, doi: 10.1016/S0140-6736(84)91816-6.

[8] N. R. Salama, M. L. Hartung, and A. Müller, 'Life in the human stomach: persistence strategies of the bacterial pathogen Helicobacter pylori', *Nature Reviews Microbiology 2013 11:6*, vol. 11, no. 6, pp. 385–399, May 2013, doi: 10.1038/nrmicro3016.

[9] B. Marshall, 'A brief history of the discovery of helicobacter pylori', *Helicobacter Pylori*, pp. 3–15, Jan. 2016, doi: 10.1007/978-4-431-55705-0_1/COVER.

[10] J. M. Pajares and J. P. Gisbert, 'Helicobacter pylori: its discovery and relevance for medicine', *Revista Española de Enfermedades Digestivas*, vol. 98, no. 10, pp. 770–785, 2006.

[11] ‘Press release: The Nobel Prize in Physiology or Medicine 2005 - NobelPrize.org’. Accessed: Jun. 20, 2024. [Online]. Available: https://www.nobelprize.org/prizes/medicine/2005/press-release/

[12] H. Yoshiyama and T. Nakazawa, ‘Unique mechanism of Helicobacter pylori for colonizing the gastric mucus’, *Microbes Infect*, vol. 2, no. 1, pp. 55–60, Jan. 2000, doi: 10.1016/S1286-4579(00)00285-9.

[13] C. A. Gonázlez and L. López-Carrillo, ‘Helicobacter pylori, nutrition and smoking interactions: Their impact in gastric carcinogenesis’, *Scand J Gastroenterol*, vol. 45, no. 1, pp. 6–14, Jan. 2010, doi: 10.3109/00365520903401959.

[14] J. A. Didonato, F. Mercurio, and M. Karin, ‘NF-κB and the link between inflammation and cancer’, *Immunol Rev*, vol. 246, no. 1, pp. 379–400, Mar. 2012, doi: 10.1111/J.1600-065X.2012.01099.X.

[15] D. Basso *et al.*, ‘Clinical Relevance of Helicobacter pylori cagA and vacA Gene Polymorphisms’, *Gastroenterology*, vol. 135, no. 1, pp. 91–99, Jul. 2008, doi: 10.1053/J.GASTRO.2008.03.041.

[16] ‘Test and treat for Helicobacter pylori (HP) in dyspepsia Quick reference guide for primary care: For consultation and local

adaptation', 2017, Accessed: Dec. 26, 2023. [Online]. Available: https://www.gov.uk/government/publications/helicobacter-pylori-diagnosis-and-treatment

[17] P. Malfertheiner *et al.*, 'Management of Helicobacter pylori infection: the Maastricht VI/Florence consensus report', *Gut*, vol. 71, pp. 1724–1762, 2022, doi: 10.1136/gutjnl-2022-327745.

[18] R. P. Allaker, K. A. Young, J. M. Hardie, P. Domizio, and N. J. Meadows, 'Prevalence of Helicobacter pylori at oral and gastrointestinal sites in children: Evidence for possible oral-to-oral transmission', 2002. doi: 10.1099/0022-1317-51-4-312.

[19] E. C. E. Gebara, C. M. Faria, C. Pannuti, L. Chehter, M. P. A. Mayer, and L. A. P. A. Lima, 'Persistence of Helicobacter pylori in the oral cavity after systemic eradication therapy', *J Clin Periodontol*, vol. 33, no. 5, pp. 329–333, May 2006, doi: 10.1111/J.1600-051X.2006.00915.X.

[20] S. Kayali *et al.*, 'Helicobacter pylori, transmission routes and recurrence of infection: state of the art.', *Acta Biomed*, vol. 89, no. 8-S, pp. 72–76, 2018, doi: 10.23750/abm.v89i8-S.7947.

[21] J. G. Kusters, A. H. M. Van Vliet, and E. J. Kuipers, 'Pathogenesis of Helicobacter pylori Infection', *Clin Microbiol Rev*, vol. 19, no. 3, p. 449, Jul. 2006, doi: 10.1128/CMR.00054-05.

[22] S. Nahar *et al.*, 'Evidence of intra-familial transmission of Helicobacter pylori by PCR-based RAPD fingerprinting in Bangladesh', *Eur J Clin Microbiol Infect Dis*, vol. 28, no. 7, pp. 767–773, Jul. 2009, doi: 10.1007/S10096-008-0699-8.

[23] T. Osaki *et al.*, 'Analysis of intra-familial transmission of Helicobacter pylori in Japanese families', *J Med Microbiol*, vol. 64, no. 1, pp. 67–73, Jan. 2015, doi: 10.1099/JMM.0.080507-0/CITE/REFWORKS.

[24] A. Savoldi, E. Carrara, D. Y. Graham, M. Conti, and E. Tacconelli, 'Prevalence of Antibiotic Resistance in Helicobacter pylori: A Systematic Review and Meta-analysis in World Health Organization Regions', *Gastroenterology*, vol. 155, no. 5, pp. 1372-1382.e17, Nov. 2018, doi: 10.1053/J.GASTRO.2018.07.007.

[25] D. G. Beevers, G. Y. H. Lip, and A. D. Blann, 'Salt intake and Helicobacter pylori infection', *J Hypertens*, vol. 22, no. 8, pp. 1475–1477, 2004, doi: 10.1097/01.HJH.0000133736.77866.77.

[26] H. Brenner, D. Rothenbacher, G. Bode, and G. Adler, ‘Relation of smoking and alcohol and coffee consumption to active Helicobacter pylori infection: cross sectional study.’, *BMJ : British Medical Journal*, vol. 315, no. 7121, p. 1489, Dec. 1997, doi: 10.1136/BMJ.315.7121.1489.

[27] J. Yu, Y. Lv, P. Yang, Y. Jiang, X. Qin, and X. Wang, ‘Alcohol increases treatment failure for Helicobacter pylori eradication in Asian populations’, *BMC Gastroenterol*, vol. 23, no. 1, pp. 1–13, Dec. 2023, doi: 10.1186/S12876-023-03002-Z/TABLES/3.

[28] T. Domanovich-Asor, H. A. Craddock, Y. Motro, B. Khalfin, A. Peretz, and J. Moran-Gilad, ‘Unraveling antimicrobial resistance in Helicobacter pylori: Global resistome meets global phylogeny’, *Helicobacter*, vol. 26, no. 2, p. e12782, Apr. 2021, doi: 10.1111/HEL.12782.

[29] H. G. Safaei, E. Rahimi, A. Zandi, and A. Rashidipour, ‘Helicobacter pylori as a zoonotic infection: the detection of H. pylori antigens in the milk and faeces of cows’, *J Res Med Sci*, vol. 16, no. 2, p. 184, 2011, Accessed: Jan. 09, 2024. [Online]. Available: /pmc/articles/PMC3214301/

[30] M. P. Dore *et al.*, ‘Isolation of Helicobacter pylori from sheep-implications for transmission to humans’, *Am J Gastroenterol*,

vol. 96, no. 5, pp. 1396–1401, May 2001, doi: 10.1111/J.1572-0241.2001.03772.X.

[31] S. Fujimori, ‘Gastric acid level of humans must decrease in the future’, *World J Gastroenterol*, vol. 26, no. 43, p. 6706, Nov. 2020, doi: 10.3748/WJG.V26.I43.6706.

[32] S. M. Tennant *et al.*, ‘Influence of Gastric Acid on Susceptibility to Infection with Ingested Bacterial Pathogens’, *Infect Immun*, vol. 76, no. 2, p. 639, Feb. 2008, doi: 10.1128/IAI.01138-07.

[33] M.-K. Shin *et al.*, ‘Characterization of Specific IgA Response to Antigenic Determinants of Helicobacter pylori Urease Encoded by ureA and ureB in Children’, *J Bacteriol Virol*, vol. 48, no. 1, pp. 14–22, 2018, doi: 10.4167/jbv.2018.48.1.14.

[34] M. Mollenhauer-Rektorschek, G. Hanauer, G. Sachs, and K. Melchers, ‘Expression of UreI is required for intragastric transit and colonization of gerbil gastric mucosa by Helicobacter pylori’, *Res Microbiol*, vol. 153, no. 10, pp. 659–666, Dec. 2002, doi: 10.1016/S0923-2508(02)01380-3.

[35] D. Y. Graham and M. Miftahussurur, ‘Helicobacter pylori urease for diagnosis of Helicobacter pylori infection: A mini review’, *J Adv Res*, vol. 13, pp. 51–57, Sep. 2018, doi: 10.1016/J.JARE.2018.01.006.

[36] S. Lindén, H. Nordman, J. Hedenbro, M. Hurtig, T. Borén, and I. Carlstedt, 'Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms', *Gastroenterology*, vol. 123, no. 6, pp. 1923–1930, Dec. 2002, doi: 10.1053/gast.2002.37076.

[37] S. D. Babu, V. Jayanthi, N. Devaraj, C. A. Reis, and H. Devaraj, 'Expression profile of mucins (MUC2, MUC5AC and MUC6) in Helicobacter pylori infected pre-neoplastic and neoplastic human gastric epithelium', *Mol Cancer*, vol. 5, no. 1, pp. 1–7, Mar. 2006, doi: 10.1186/1476-4598-5-10/TABLES/2.

[38] J. H. B. Van de Bovenkamp *et al.*, 'The MUC5AC Glycoprotein is the Primary Receptor for Helicobacter pylori in the Human Stomach', *Helicobacter*, vol. 8, no. 5, pp. 521–532, Oct. 2003, doi: 10.1046/J.1523-5378.2003.00173.X.

[39] D. Doohan, Y. A. A. Rezkitha, L. A. Waskito, Y. Yamaoka, and M. Miftahussurur, 'Helicobacter pylori BabA–SabA Key Roles in the Adherence Phase: The Synergic Mechanism for Successful Colonization and Disease Development', *Toxins 2021, Vol. 13, Page 485*, vol. 13, no. 7, p. 485, Jul. 2021, doi: 10.3390/TOXINS13070485.

[40] R. R. Caston *et al.*, 'Effect of environmental salt concentration on the Helicobacter pylori exoproteome', *J Proteomics*, vol.

202, p. 103374, Jun. 2019, doi: 10.1016/J.JPROT.2019.05.002.

[41] X. Wu, L. Chen, J. Cheng, J. Qian, Z. Fang, and J. Wu, ‘Effect of Dietary Salt Intake on Risk of Gastric Cancer: A Systematic Review and Meta-Analysis of Case-Control Studies’, *Nutrients*, vol. 14, no. 20, Oct. 2022, doi: 10.3390/NU14204260/S1.

[42] K. M. Ottemann and A. C. Lowenthal, ‘Helicobacter pylori uses motility for initial colonization and to attain robust infection’, *Infect Immun*, vol. 70, no. 4, pp. 1984–1990, 2002, doi: 10.1128/IAI.70.4.1984-1990.2002/ASSET/418A5035-4FA1-451E-AE17-E976CA26F37E/ASSETS/GRAPHIC/II0420887004.JPEG.

[43] K. A. Eaton, D. R. Morgan, and S. Krakowka, ‘Motility as a factor in the colonisation of gnotobiotic piglets by Helicobacter pylori’, *J Med Microbiol*, vol. 37, no. 2, pp. 123–127, Aug. 1992, doi: 10.1099/00222615-37-2-123/CITE/REFWORKS.

[44] P. W. O’ Toole, M. C. Lane, and S. Porwollik, ‘Helicobacter pylori motility’, *Microbes Infect*, vol. 2, no. 10, pp. 1207–1214, Aug. 2000, doi: 10.1016/S1286-4579(00)01274-0.

[45] C. Dunne, B. Dolan, and M. Clyne, ‘Factors that mediate colonization of the human stomach by Helicobacter pylori’, *World Journal of Gastroenterology : WJG*, vol. 20, no. 19, p. 5610, May 2014, doi: 10.3748/WJG.V20.I19.5610.

[46] L. K. Sycuro *et al.*, ‘Peptidoglycan crosslinking relaxation promotes Helicobacter pylori’s helical shape and stomach colonization’, *Cell*, vol. 141, no. 5, pp. 822–833, 2010, doi: 10.1016/J.CELL.2010.03.046.

[47] K. S. Johnson and K. M. Ottemann, ‘Colonization, localization, and inflammation: the roles of H. pylori chemotaxis in vivo’, *Curr Opin Microbiol*, vol. 41, pp. 51–57, Feb. 2018, doi: 10.1016/J.MIB.2017.11.019.

[48] N. Navabi, M. E. V. Johansson, S. Raghavan, and S. K. Lindéna, ‘Helicobacter pylori infection impairs the mucin production rate and turnover in the murine gastric mucosa’, *Infect Immun*, vol. 81, no. 3, pp. 829–837, Mar. 2013, doi: 10.1128/IAI.01000-12/ASSET/9AFD86DE-7E21-486E-B6C9-6A81E01CC259/ASSETS/GRAPHIC/ZII9990900230007.JPEG.

[49] H. J. Windle, Á. Fox, D. N. Eidhin, and D. Kelleher, ‘The Thioredoxin System of Helicobacter pylori’, *Journal of Biological Chemistry*, vol. 275, no. 7, pp. 5081–5089, Feb. 2000, doi: 10.1074/JBC.275.7.5081.

[50] M. D. Burkitt, C. A. Duckworth, J. M. Williams, and D. M. Pritchard, ‘Helicobacter pylori-induced gastric pathology: insights from in vivo and ex vivo models.’, *Dis Model Mech*, vol. 10, no. 2, pp. 89–104, Feb. 2017, doi: 10.1242/DMM.027649.

[51] J. A. Bugaytsova *et al.*, ‘Adaption of Helicobacter pylori to Chronic Infection and Gastric Disease by pH-Responsive BabA-Mediated Adherence’, *Cell Host Microbe*, vol. 21, no. 3, p. 376, Mar. 2017, doi: 10.1016/J.CHOM.2017.02.013.

[52] J. H. B. Shuman *et al.*, ‘Remodeling of the gastric environment in Helicobacter pylori-induced atrophic gastritis’, *mSystems*, Dec. 2023, doi: 10.1128/MSYSTEMS.01098-23.

[53] H. Ubukata, H. Nagata, T. Tabuchi, S. Konishi, T. Kasuga, and T. Tabuchi, ‘Why is the coexistence of gastric cancer and duodenal ulcer rare? Examination of factors related to both gastric cancer and duodenal ulcer’, *Gastric Cancer*, vol. 14, no. 1, pp. 4–12, Mar. 2011, doi: 10.1007/S10120-011-0005-9/FIGURES/5.

[54] T. Foukakis, L. Lundell, M. Gubanski, and P. A. Lind, ‘Advances in the treatment of patients with gastric adenocarcinoma’, *Acta Oncol (Madr)*, vol. 46, no. 3, pp. 277–285, 2007, doi: 10.1080/02841860701218634.

[55] J. W. Seo, J. Y. Park, T. S. Shin, and J. G. Kim, ‘The analysis of virulence factors and antibiotic resistance between Helicobacter pylori strains isolated from gastric antrum and body’, *BMC Gastroenterol*, vol. 19, no. 1, pp. 1–8, Aug. 2019, doi: 10.1186/S12876-019-1062-5/TABLES/3.

[56] D. J. Wilkinson, B. Dickins, K. Robinson, and J. A. Winter, ‘Genomic diversity of Helicobacter pylori populations from different regions of the human stomach’, *Gut Microbes*, vol. 14, no. 1, Dec. 2022, doi: 10.1080/19490976.2022.2152306.

[57] K. Ben Mansour *et al.*, ‘Multiple and mixed Helicobacter pylori infections: Comparison of two epidemiological situations in Tunisia and France’, *Infection, Genetics and Evolution*, vol. 37, pp. 43–48, Jan. 2016, doi: 10.1016/J.MEEGID.2015.10.028.

[58] B. V. Arévalo-Jaimes, D. F. Rojas-Rengifo, C. A. Jaramillo, B. M. De Molano, J. F. Vera-Chamorro, and M. Del Pilar Delgado, ‘Genotypic determination of resistance and heteroresistance to clarithromycin in Helicobacter pylori isolates from antrum and corpus of Colombian symptomatic patients’, *BMC Infect Dis*, vol. 19, no. 1, pp. 1–8, Jun. 2019, doi: 10.1186/S12879-019-4178-X/FIGURES/4.

[59] Y. Sun and J. Zhang, ‘Helicobacter pylori recrudescence and its influencing factors’, *J Cell Mol Med*, vol. 23, no. 12, pp. 7919–7925, Dec. 2019, doi: 10.1111/JCMM.14682.

[60] V. Herrera and J. Parsonnet, ‘Helicobacter pylori and gastric adenocarcinoma’, *Clinical Microbiology and Infection*, vol. 15, no. 11, pp. 971–976, Nov. 2009, doi: 10.1111/J.1469-0691.2009.03031.X.

[61] D. B. Polk and R. M. Peek, ‘Helicobacter pylori: gastric cancer and beyond’, *Nat Rev Cancer*, vol. 10, no. 6, pp. 403–414, Jun. 2010, doi: 10.1038/nrc2857.

[62] L. P. Carcas, ‘Gastric cancer review’, *J Carcinog*, vol. 13, 2014, doi: 10.4103/1477-3163.146506.

[63] J. S. Muhammad, T. Sugiyama, and S. F. Zaidi, ‘Gastric pathophysiological Ins and outs of helicobacter pylori: A review’, 2013.

[64] P. Rawla and A. Barsouk, ‘Epidemiology of gastric cancer: global trends, risk factors and prevention.’, *Prz Gastroenterol*, vol. 14, no. 1, pp. 26–38, 2019, doi: 10.5114/pg.2018.80001.

[65] A. Zullo, C. Hassan, L. Ridola, A. Repici, R. Manta, and A. Andriani, ‘Gastric MALT lymphoma: old and new insights’, *Ann Gastroenterol*, vol. 27, no. 1, p. 27, 2014.

[66] J. J. Y. Sung, E. J. Kuipers, and H. B. El-Serag, ‘Systematic review: The global incidence and prevalence of peptic ulcer disease’, *Aliment Pharmacol Ther*, vol. 29, no. 9, pp. 938–946, May 2009, doi: 10.1111/j.1365-2036.2009.03960.x.

[67] P. Tomtitchong, B. Siribumrungwong, R.-K. Vilaichone, P. Kasetsuwan, N. Matsukura, and N. Chaiyakunapruk, ‘Systematic Review and Meta-Analysis: Helicobacter pylori

Eradication Therapy After Simple Closure of Perforated Duodenal Ulcer', *Helicobacter*, vol. 17, no. 2, pp. 148–152, Apr. 2012, doi: 10.1111/j.1523-5378.2011.00928.x.

[68] J. L. Rhead *et al.*, 'A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer.', *Gastroenterology*, vol. 133, no. 3, pp. 926–36, Sep. 2007, doi: 10.1053/j.gastro.2007.06.056.

[69] A. pormohammad, R. Ghotaslo, H. E. Leylabadlo, M. J. Nasiri, H. Dabiri, and A. Hashemi, 'Risk of gastric cancer in association with Helicobacter pylori different virulence factors: A systematic review and meta-analysis', *Microb Pathog*, vol. 118, pp. 214–219, May 2018, doi: 10.1016/j.micpath.2018.03.004.

[70] X.-Y. Zhang, P.-Y. Zhang, and M. Aboul-Soud, 'From inflammation to gastric cancer: Role of Helicobacter pylori (Review)', *Oncol Lett*, vol. 13, no. 2, pp. 543–548, Feb. 2017, Accessed: Feb. 20, 2024. [Online]. Available: https://www.spandidos-publications.com/10.3892/ol.2016.5506

[71] S. M. Smith, 'Role of Toll-like receptors in Helicobacter pylori infection and immunity', *World J Gastrointest Pathophysiol*, vol. 5, no. 3, p. 133, Aug. 2014, doi: 10.4291/WJGP.V5.I3.133.

[72] E. M. El-Omar, M. T. Ng, and G. L. Hold, ‘Polymorphisms in Toll-like receptor genes and risk of cancer’, *Oncogene 2008 27:2*, vol. 27, no. 2, pp. 244–252, Jan. 2008, doi: 10.1038/sj.onc.1210912.

[73] Y. Hu, J. H. Wan, X. Y. Li, Y. Zhu, D. Y. Graham, and N. H. Lu, ‘Systematic review with meta-analysis: the global recurrence rate of Helicobacter pylori’, *Aliment Pharmacol Ther*, vol. 46, no. 9, pp. 773–779, Nov. 2017, doi: 10.1111/APT.14319.

[74] R. Nomura, Y. Ogaya, S. Matayoshi, Y. Morita, and K. Nakano, ‘Molecular and clinical analyses of Helicobacter pylori colonization in inflamed dental pulp’, *BMC Oral Health*, vol. 18, no. 1, pp. 1–9, Apr. 2018, doi: 10.1186/S12903-018-0526-2/FIGURES/4.

[75] A. Román-Román, S. Giono-Cerezo, M. Camorlinga-Ponce, D. N. Martínez-Carrillo, S. Loaiza-Loeza, and G. Fernández-Tilapa, ‘vacA genotypes of Helicobacter pylori in the oral cavity and stomach of patients with chronic gastritis and gastric ulcer’, *Enferm Infecc Microbiol Clin*, vol. 31, no. 3, pp. 130–135, Mar. 2013, doi: 10.1016/J.EIMC.2012.09.002.

[76] Q. Song, T. Lange, A. Spahr, G. Adler, and G. Bode, ‘Characteristic distribution pattern of Helicobacter pylori in dental plaque and saliva detected with nested PCR’, *J Med*

Microbiol, vol. 49, no. 4, pp. 349–353, 2000, doi: 10.1099/0022-1317-49-4-349.

[77] Wang *et al.*, 'Oral Helicobacter pylori, its relationship to successful eradication of gastric H. pylori and saliva culture confirmation', *Journal of Physiology and Pharmacology*, vol. 65, no. 4, pp. 559–566, 2014, Accessed: Jan. 07, 2024. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/25179088/

[78] L. Zhang, X. Chen, B. Ren, X. Zhou, and L. Cheng, 'Helicobacter pylori in the Oral Cavity: Current Evidence and Potential Survival Strategies', *Int J Mol Sci*, vol. 23, no. 21, Nov. 2022, doi: 10.3390/IJMS232113646.

[79] P. Malfertheiner *et al.*, 'Management of Helicobacter pylori infection: the Maastricht VI/Florence consensus report', *Gut*, vol. 71, no. 9, pp. 1724–1762, Sep. 2022, doi: 10.1136/GUTJNL-2022-327745.

[80] M. Nocon *et al.*, 'Efficacy and cost-effectiveness of the 13C-urea breath test as the primary diagnostic investigation for the detection of Helicobacter pylori infection compared to invasive and non-invasive diagnostic tests', *GMS Health Innov Technol*, vol. 21, no. 5, pp. 1–12, Oct. 2009.

[81] L. Mazzei, F. Musiani, and S. Ciurli, *Helicobacter pylori: Physiology and Genetics - Urease*, no. 10. ASM Press, 2001. doi: 10.1039/9781788010580-00060.

[82] A. Berger, 'How does it work?: Helicobacter pylori breath tests', *BMJ : British Medical Journal*, vol. 324, no. 7348, p. 1263, May 2002, doi: 10.1136/BMJ.324.7348.1263.

[83] Y. K. Wang *et al.*, 'Diagnosis of Helicobacter pylori infection: Current options and developments', *World Journal of Gastroenterology : WJG*, vol. 21, no. 40, p. 11221, Oct. 2015, doi: 10.3748/WJG.V21.I40.11221.

[84] A. D. Pronovost, S. L. Rose, J. W. Pawlak, H. Robin, and A. R. Schneider3, 'Evaluation of a New Immunodiagnostic Assay for Helicobacter pyloni Antibody Detection: Correlation with Histopathological and Microbiological Results', *J Clin Microbiol*, vol. 32, no. 1, pp. 46–50, 1994.

[85] E. Qiu, Z. Li, and S. Han, 'Methods for detection of Helicobacter pylori from stool sample: current options and developments', *Brazilian Journal of Microbiology*, vol. 52, no. 4, pp. 2057–2062, Dec. 2021, doi: 10.1007/S42770-021-00589-X/TABLES/1.

[86] X. Zhou, J. Su, G. Xu, and G. Zhang, 'Accuracy of stool antigen test for the diagnosis of Helicobacter pylori infection in children: A meta-analysis', *Clin Res Hepatol Gastroenterol*,

vol. 38, no. 5, pp. 629–638, Oct. 2014, doi: 10.1016/J.CLINRE.2014.02.001.

[87] J. M. K. da Silva-Etto, R. Mattar, C. A. Villares-Lopes, S. B. Marques, and F. J. Carrilho, 'Evaluation of diagnostic accuracy of two rapid stool antigen tests using an immunochromatographic assay to detect Helicobacter pylori', *Clin Biochem*, vol. 50, no. 16–17, pp. 959–962, Nov. 2017, doi: 10.1016/J.CLINBIOCHEM.2017.05.005.

[88] T. Shimoyama, 'Stool antigen tests for the management of Helicobacter pylori infection', *World Journal of Gastroenterology : WJG*, vol. 19, no. 45, p. 8188, Dec. 2013, doi: 10.3748/WJG.V19.I45.8188.

[89] D. Vaira *et al.*, 'The stool antigen test for detection of Helicobacter pylori after eradication therapy', *Ann Intern Med*, vol. 136, no. 4, pp. 280–287, Feb. 2002, doi: 10.7326/0003-4819-136-4-200202190-00007.

[90] M. Friedt and S. Welsch, 'An update on pediatric endoscopy', *Eur J Med Res*, vol. 18, no. 1, p. 24, 2013, doi: 10.1186/2047-783X-18-24.

[91] C. Rescalvo-Casas *et al.*, 'Comparison of chemiluminiscence versus lateral flow assay for the detection of Helicobacter pylori antigen in human fecal samples', *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 42, no. 8,

pp. 959–962, Aug. 2023, doi: 10.1007/S10096-023-04624-7/TABLES/2.

[92] T. Kakiuchi *et al.*, 'Assessment of a novel method to detect clarithromycin-resistant Helicobacter pylori using a stool antigen test reagent', *BMC Gastroenterol*, vol. 20, no. 1, pp. 1–6, Dec. 2020, doi: 10.1186/S12876-020-01549-9/TABLES/3.

[93] D. E. Brennan *et al.*, 'Molecular detection of Helicobacter pylori antibiotic resistance in stool vs biopsy samples', *World J Gastroenterol*, vol. 22, no. 41, p. 9214, Nov. 2016, doi: 10.3748/WJG.V22.I41.9214.

[94] R. J. Gong, C. X. Xu, H. Li, and X. M. Liu, 'Polymerase chain reaction-based tests for detecting Helicobacter pylori clarithromycin resistance in stool samples: A meta-analysis', *World J Clin Cases*, vol. 9, no. 1, p. 133, Jan. 2021, doi: 10.12998/WJCC.V9.I1.133.

[95] D. Vaira *et al.*, 'Review article:invasive and non-invasive tests for Helicobacter pylori infection', *Aliment Pharmacol Ther*, vol. 14 Suppl 3, no. s3, pp. 13–22, Oct. 2000, doi: 10.1046/J.1365-2036.2000.00096.X.

[96] P. Yao *et al.*, 'Helicobacter pylori multiplex serology and risk of non-cardia and cardia gastric cancer: a case-cohort study and

meta-analysis', *Int J Epidemiol*, vol. 52, no. 4, pp. 1197–1208, Aug. 2023, doi: 10.1093/IJE/DYAD007.

[97] J. P. Gisbert and J. M. Pajares, 'Review article: 13C-urea breath test in the diagnosis of Helicobacter pylori infection – a critical review', *Aliment Pharmacol Ther*, vol. 20, no. 10, pp. 1001–1017, Nov. 2004, doi: 10.1111/J.1365-2036.2004.02203.X.

[98] A. I. Lopes, F. F. Vale, and M. Oleastro, 'Helicobacter pylori infection - recent developments in diagnosis', *World Journal of Gastroenterology : WJG*, vol. 20, no. 28, p. 9299, Jul. 2014, doi: 10.3748/WJG.V20.I28.9299.

[99] J. J. Redondo, P. M. Keller, R. Zbinden, and K. Wagner, 'A novel RT-PCR for the detection of Helicobacter pylori and identification of clarithromycin resistance mediated by mutations in the 23S rRNA gene', *Diagn Microbiol Infect Dis*, vol. 90, no. 1, pp. 1–6, Jan. 2018, doi: 10.1016/J.DIAGMICROBIO.2017.09.014.

[100] D. S. Bordin, I. N. Voynovan, D. N. Andreev, and I. V. Maev, 'Current Helicobacter pylori Diagnostics', *Diagnostics 2021, Vol. 11, Page 1458*, vol. 11, no. 8, p. 1458, Aug. 2021, doi: 10.3390/DIAGNOSTICS11081458.

[101] J. K. Y. Hooi *et al.*, 'Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis',

Gastroenterology, vol. 153, no. 2, pp. 420–429, Aug. 2017, doi: 10.1053/j.gastro.2017.04.022.

[102] S. Smith, M. Fowora, and R. Pellicano, 'Infections with Helicobacter pylori and challenges encountered in Africa', *World J Gastroenterol*, vol. 25, no. 25, p. 3183, Jul. 2019, doi: 10.3748/WJG.V25.I25.3183.

[103] A. Nyerere Kimang'a, G. Revathi, S. Kariuki, S. Sayed, and S. Devani, 'Helicobacter pylori: Prevalence and antibiotic susceptibility among Kenyans', South African Medical Journal. Accessed: Jan. 02, 2024. [Online]. Available: https://www.scielo.org.za/scielo.php?script=sci_arttext&pid=S0256-95742010000100021

[104] S. Z. Bakhti, S. Latifi-Navid, and R. Safaralizadeh, 'Helicobacter pylori-related risk predictors of gastric cancer: The latest models, challenges, and future prospects', *Cancer Med*, vol. 9, no. 13, pp. 4808–4822, Jul. 2020, doi: 10.1002/CAM4.3068.

[105] W. L. Chang, Y. C. Yeh, and B. S. Sheu, 'The impacts of H. pylori virulence factors on the development of gastroduodenal diseases', *J Biomed Sci*, vol. 25, no. 1, pp. 1–9, Sep. 2018, doi: 10.1186/S12929-018-0466-9/TABLES/3.

[106] J. Y. Park, D. Forman, L. A. Waskito, Y. Yamaoka, and J. E. Crabtree, 'Epidemiology of Helicobacter pylori and CagA-

Positive Infections and Global Variations in Gastric Cancer', *Toxins 2018, Vol. 10, Page 163*, vol. 10, no. 4, p. 163, Apr. 2018, doi: 10.3390/TOXINS10040163.

[107] D. Palli *et al.*, 'CagA+ Helicobacter pylori infection and gastric cancer risk in the EPIC-EURGAST study', *Int J Cancer*, vol. 120, no. 4, pp. 859–867, Feb. 2007, doi: 10.1002/IJC.22435.

[108] B. Linz *et al.*, 'An African origin for the intimate association between humans and Helicobacter pylori', *Nature*, vol. 445, no. 7130, pp. 915–918, Feb. 2007, doi: 10.1038/NATURE05562.

[109] M. Zamani *et al.*, 'Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection', *Aliment Pharmacol Ther*, vol. 47, no. 7, pp. 868–876, Apr. 2018, doi: 10.1111/APT.14561.

[110] J. Bastos *et al.*, 'Sociodemographic determinants of prevalence and incidence of Helicobacter pylori infection in Portuguese adults', *Helicobacter*, vol. 18, no. 6, pp. 413–422, Dec. 2013, doi: 10.1111/HEL.12061.

[111] A. Ibrahim, S. Morais, A. Ferro, N. Lunet, and B. Peleteiro, 'Sex-differences in the prevalence of Helicobacter pylori infection in pediatric and adult populations: Systematic review and meta-analysis of 244 studies', *Digestive and Liver*

Disease, vol. 49, no. 7, pp. 742–749, Jul. 2017, doi: 10.1016/j.dld.2017.03.019.

[112] N. D. Freedman, M. H. Derakhshan, C. C. Abnet, A. Schatzkin, A. R. Hollenbeck, and K. E. L. McColl, 'Male predominance of upper gastrointestinal adenocarcinoma cannot be explained by differences in tobacco smoking in men versus women', *Eur J Cancer*, vol. 46, no. 13, p. 2473, Sep. 2010, doi: 10.1016/J.EJCA.2010.05.005.

[113] K. Robinson, R. H. Argent, and J. C. Atherton, 'The inflammatory and immune response to Helicobacter pylori infection', *Best Pract Res Clin Gastroenterol*, vol. 21, no. 2, pp. 237–259, Apr. 2007, doi: 10.1016/J.BPG.2007.01.001.

[114] M. El Khadir *et al.*, 'VacA and CagA Status as Biomarker of Two Opposite End Outcomes of Helicobacter pylori Infection (Gastric Cancer and Duodenal Ulcer) in a Moroccan Population', *PLoS One*, vol. 12, no. 1, p. 170616, Jan. 2017, doi: 10.1371/JOURNAL.PONE.0170616.

[115] D. P. Letley and J. C. Atherton, 'Natural Diversity in the N Terminus of the Mature Vacuolating Cytotoxin of Helicobacter pylori Determines Cytotoxin Activity', *J Bacteriol*, vol. 182, no. 11, p. 3278, Jun. 2000, doi: 10.1128/JB.182.11.3278-3280.2000.

[116] J. C. Atherton, P. Cao, R. M. Peek, M. K. R. Tummuru, M. J. Blaser, and T. L. Cover, 'Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration', *J Biol Chem*, vol. 270, no. 30, pp. 17771–17777, Jul. 1995, doi: 10.1074/JBC.270.30.17771.

[117] E. E. Hennig, J. M. Allen, and T. L. Cover, 'Multiple Chromosomal Loci for the babA Gene in Helicobacter pylori', *Infect Immun*, vol. 74, no. 5, p. 3046, May 2006, doi: 10.1128/IAI.74.5.3046-3051.2006.

[118] R. H. Argent, R. J. Thomas, D. P. Letley, M. G. Rittig, K. R. Hardie, and J. C. Atherton, 'Functional association between the Helicobacter pylori virulence factors VacA and CagA', *J Med Microbiol*, vol. 57, no. 2, pp. 145–150, Feb. 2008, doi: 10.1099/JMM.0.47465-0/CITE/REFWORKS.

[119] J. A. Winter *et al.*, 'A Role for the Vacuolating Cytotoxin, VacA, in Colonization and Helicobacter pylori–Induced Metaplasia in the Stomach', *J Infect Dis*, vol. 210, no. 6, pp. 954–963, Sep. 2014, doi: 10.1093/INFDIS/JIU154.

[120] J. C. Atherton *et al.*, 'Vacuolating cytotoxin (vacA) alleles of Helicobacter pylori comprise two geographically widespread types, m1 and m2, and have evolved through limited

recombination', *Curr Microbiol*, vol. 39, no. 4, pp. 211–218, 1999, doi: 10.1007/S002849900447/METRICS.

[121] C. Pagliaccia *et al.*, 'The m2 form of the Helicobacter pylori cytotoxin has cell type-specific vacuolating activity', *Proc Natl Acad Sci U S A*, vol. 95, no. 17, p. 10212, Aug. 1998, doi: 10.1073/PNAS.95.17.10212.

[122] S. Latifi-Navid *et al.*, 'Helicobacter pylori vacA d1/-i1 Genotypes and Geographic Differentiation between High and Low Incidence Areas of Gastric Cancer in Iran.', *Arch Iran Med*, vol. 16, no. 6, pp. 330–337, 2013, Accessed: Dec. 30, 2023. [Online]. Available: https://openurl.ebsco.com/EPDB%3Agcd%3A4%3A13185364/detailv2?sid=ebsco%3Aplink%3Ascholar&id=ebsco%3Agcd%3A90310584&crl=c

[123] T. L. Cover, R. L. Holland, and S. R. Blanke, 'Helicobacter pylori vacuolating toxin', *Helicobacter pylori Research: From Bench to Bedside*, pp. 113–142, Jan. 2016, doi: 10.1007/978-4-431-55936-8_5/COVER.

[124] N. J. Foegeding, R. R. Caston, M. S. McClain, M. D. Ohi, and T. L. Cover, 'An Overview of Helicobacter pylori VacA Toxin Biology', *Toxins 2016, Vol. 8, Page 173*, vol. 8, no. 6, p. 173, Jun. 2016, doi: 10.3390/TOXINS8060173.

[125] D. P. Letley, J. L. Rhead, K. Bishop, and J. C. Atherton, 'Paired cysteine residues are required for high levels of the Helicobacter pylori autotransporter VacA', *Microbiology (N Y)*, vol. 152, no. 5, pp. 1319–1325, May 2006, doi: 10.1099/MIC.0.28548-0/CITE/REFWORKS.

[126] S. L. Palframan, T. Kwok, and K. Gabriel, 'Vacuolating cytotoxin A (VacA), a key toxin for Helicobacter pylori pathogenesis.', *Front Cell Infect Microbiol*, vol. 2, p. 92, Jul. 2012, doi: 10.3389/FCIMB.2012.00092/BIBTEX.

[127] G. Zanotti and L. Cendron, 'Structural and functional aspects of the Helicobacter pylori secretome', *World Journal of Gastroenterology : WJG*, vol. 20, no. 6, p. 1402, Feb. 2014, doi: 10.3748/WJG.V20.I6.1402.

[128] L. Chatre *et al.*, 'Helicobacter pylori targets mitochondrial import and components of mitochondrial DNA replication machinery through an alternative VacA-dependent and a VacA-independent mechanisms', *Scientific Reports 2017 7:1*, vol. 7, no. 1, pp. 1–14, Nov. 2017, doi: 10.1038/s41598-017-15567-3.

[129] D. A. Israel *et al.*, 'Helicobacter pylori genetic diversity within the gastric niche of a single human host', *Proc Natl Acad Sci U S A*, vol. 98, no. 25, pp. 14625–14630, Dec. 2001, doi: 10.1073/PNAS.251551698/SUPPL_FILE/5516SUPPTEXT.TSV.

[130] M. Sugimoto, M. R. Zali, and Y. Yamaoka, ‘The association of vacA genotypes and Helicobacter pylori-related gastroduodenal diseases in the Middle East’, *Eur J Clin Microbiol Infect Dis*, vol. 28, no. 10, pp. 1227–1236, Oct. 2009, doi: 10.1007/S10096-009-0772-Y.

[131] A. Tohidpour, ‘CagA-mediated pathogenesis of Helicobacter pylori’, *Microb Pathog*, vol. 93, pp. 44–55, Apr. 2016, doi: 10.1016/J.MICPATH.2016.01.005.

[132] J. M. Noto and R. M. Peek, ‘The Helicobacter pylori cag Pathogenicity Island’, *Methods in Molecular Biology*, vol. 921, pp. 41–50, 2012, doi: 10.1007/978-1-62703-005-2_7.

[133] M. Hatakeyama, ‘Structure and function of Helicobacter pylori CagA, the first-identified bacterial protein involved in human cancer’, *Proc Jpn Acad Ser B Phys Biol Sci*, vol. 93, no. 4, p. 196, Apr. 2017, doi: 10.2183/PJAB.93.013.

[134] W. Fischer, ‘Assembly and molecular mode of action of the Helicobacter pylori Cag type IV secretion apparatus’, *FEBS J*, vol. 278, no. 8, pp. 1203–1212, 2011, doi: 10.1111/J.1742-4658.2011.08036.X.

[135] C. C. Chang, W. S. Kuo, Y. C. Chen, C. L. Perng, H. J. Lin, and Y. H. Ou, ‘Fragmentation of CagA Reduces Hummingbird Phenotype Induction by Helicobactor pylori’, *PLoS One*, vol. 11, no. 3, Mar. 2016, doi: 10.1371/JOURNAL.PONE.0150061.

[136] S. Backert, N. Tegtmeyer, and M. Selbach, 'The Versatility of Helicobacter pylori CagA Effector Protein Functions: The Master Key Hypothesis', *Helicobacter*, vol. 15, no. 3, pp. 163–176, Jun. 2010, doi: 10.1111/J.1523-5378.2010.00759.X.

[137] R. J. Gorrell *et al.*, 'A novel NOD1- and CagA-independent pathway of interleukin-8 induction mediated by the Helicobacter pylori type IV secretion system', *Cell Microbiol*, vol. 15, no. 4, pp. 554–570, Apr. 2013, doi: 10.1111/CMI.12055.

[138] M. Tafreshi *et al.*, 'Helicobacter pylori type IV secretion system and its adhesin subunit, CagL, mediate potent inflammatory responses in primary human endothelial cells', *Front Cell Infect Microbiol*, vol. 8, no. FEB, p. 270756, Feb. 2018, doi: 10.3389/FCIMB.2018.00022/BIBTEX.

[139] J. A. McCubrey *et al.*, 'Roles of the Raf/MEK/ERK Pathway in Cell Growth, Malignant Transformation and Drug Resistance', *Biochim Biophys Acta*, vol. 1773, no. 8, p. 1263, Aug. 2007, doi: 10.1016/J.BBAMCR.2006.10.001.

[140] T. Kwok *et al.*, 'Helicobacter exploits integrin for type IV secretion and kinase activation', *Nature 2007 449:7164*, vol. 449, no. 7164, pp. 862–866, Oct. 2007, doi: 10.1038/nature06187.

[141] J. L. Snider, C. Allison, B. H. Bellaire, R. L. Ferrero, and J. A. Cardelli, 'The β1 Integrin Activates JNK Independent of CagA, and JNK Activation Is Required for Helicobacter pylori CagA+-induced Motility of Gastric Cancer Cells', *Journal of Biological Chemistry*, vol. 283, no. 20, pp. 13952–13963, May 2008, doi: 10.1074/JBC.M800289200.

[142] M. Buß *et al.*, 'Specific high affinity interaction of Helicobacter pylori CagL with integrin αVβ6 promotes type IV secretion of CagA into human cells', *FEBS J*, vol. 286, no. 20, pp. 3980–3997, Oct. 2019, doi: 10.1111/FEBS.14962.

[143] M. Hatakeyama and H. Higashi, 'Helicobacter pylori CagA: a new paradigm for bacterial carcinogenesis', *Cancer Sci*, vol. 96, no. 12, pp. 835–843, Dec. 2005, doi: 10.1111/J.1349-7006.2005.00130.X.

[144] M. Khaledi *et al.*, 'Determination of CagA EPIYA motif in Helicobacter pylori strains isolated from patients with digestive disorder', *Heliyon*, vol. 6, no. 9, p. e04971, Sep. 2020, doi: 10.1016/J.HELIYON.2020.E04971.

[145] S. K. Mitra, D. A. Hanson, and D. D. Schlaepfer, 'Focal adhesion kinase: in command and control of cell motility', *Nature Reviews Molecular Cell Biology 2005 6:1*, vol. 6, no. 1, pp. 56–68, Jan. 2005, doi: 10.1038/nrm1549.

[146] S. S. Duncan, P. L. Valk, C. L. Shaffer, S. R. Bordenstein, and T. L. Cover, 'J-Western forms of helicobacter pylori caga constitute a distinct phylogenetic group with a widespread geographic distribution', *J Bacteriol*, vol. 194, no. 6, pp. 1593–1604, Mar. 2012, doi: 10.1128/JB.06340-11.

[147] M. El Khadir *et al.*, 'Helicobacter pylori CagA EPIYA-C motifs and gastric diseases in Moroccan patients', *Infection, Genetics and Evolution*, vol. 66, pp. 120–129, Dec. 2018, doi: 10.1016/J.MEEGID.2018.09.015.

[148] Y. Yamaoka, 'Mechanisms of disease: Helicobacter pylori virulence factors', *Nat Rev Gastroenterol Hepatol*, vol. 7, no. 11, p. 629, Nov. 2010, doi: 10.1038/NRGASTRO.2010.154.

[149] A. M. D. Machado *et al.*, 'Helicobacter pylori infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells', *Clinical Cancer Research*, vol. 15, no. 9, pp. 2995–3002, May 2009, doi: 10.1158/1078-0432.CCR-08-2686/346641/P/HELICOBACTER-PYLORI-INFECTION-INDUCES-GENETIC.

[150] B. Dörflinger *et al.*, 'Mitochondria supply sub-lethal signals for cytokine secretion and DNA-damage in H. pylori infection', *Cell Death & Differentiation 2022 29:11*, vol. 29, no. 11, pp. 2218–2232, May 2022, doi: 10.1038/s41418-022-01009-9.

[151] M. Abdullah, L. K. Greenfield, D. Bronte-Tinkew, M. I. Capurro, D. Rizzuti, and N. L. Jones, ‘VacA promotes CagA accumulation in gastric epithelial cells during Helicobacter pylori infection’, *Scientific Reports 2019 9:1*, vol. 9, no. 1, pp. 1–9, Jan. 2019, doi: 10.1038/s41598-018-37095-4.

[152] D. Boltin *et al.*, ‘Trends in secondary antibiotic resistance of Helicobacter pylori from 2007 to 2014: has the tide turned?’, *J Clin Microbiol*, vol. 53, no. 2, pp. 522–7, Feb. 2015, doi: 10.1128/JCM.03001-14.

[153] S. Shah, E. Hubscher, C. Pelletier, R. Jacob, L. Vinals, and R. Yadlapati, ‘Helicobacter pylori infection treatment in the United States: clinical consequences and costs of eradication treatment failure’, *Expert Rev Gastroenterol Hepatol*, vol. 16, no. 4, pp. 341–357, Apr. 2022, doi: 10.1080/17474124.2022.2056015.

[154] J. P. A. O’connor, I. Taneike, and C. O’morain, ‘Improving compliance with Helicobacter pylori eradication therapy: When and how?’, *Therap Adv Gastroenterol*, vol. 2, no. 5, pp. 273–279, 2009, doi: 10.1177/1756283X09337342.

[155] M. Lee, J. A. Kemp, A. Canning, C. Egan, G. Tataronis, and F. A. Farraye, ‘A Randomized Controlled Trial of an Enhanced Patient Compliance Program for Helicobacter pylori Therapy’,

Arch Intern Med, vol. 159, no. 19, pp. 2312–2316, Oct. 1999, doi: 10.1001/ARCHINTE.159.19.2312.

[156] P. Malfertheiner, 'Compliance, Adverse Events and Antibiotic Resistance in Helicobacter pylori Treatment', *Scand J Gastroenterol*, vol. 28, no. S196, pp. 34–37, 1993, doi: 10.3109/00365529309098341.

[157] H. Lu, W. Zhang, and D. Y. Graham, 'Bismuth-containing quadruple therapy for Helicobacter pylori: Lessons from China', *Eur J Gastroenterol Hepatol*, vol. 25, no. 10, pp. 1134–1140, Oct. 2013, doi: 10.1097/MEG.0B013E3283633B57.

[158] C. Alba, A. Blanco, and T. Alarcón, 'Antibiotic resistance in Helicobacter pylori', *Curr Opin Infect Dis*, vol. 30, no. 5, pp. 489–497, Oct. 2017, doi: 10.1097/QCO.0000000000000396.

[159] C. G. Sinnett, D. P. Letley, and G. L. Narayanan, 'Helicobacter pylori vacA transcription is genetically-determined and stratifies the level of human gastric inflammation and atrophy', *J Clin Pathol*, 2016, doi: 10.1136/jclinpath-2016-203641.

[160] M. F. Dixon *et al.*, 'Classification and Grading of Gastritis', *Am J Surg Pathol*, vol. 20, no. 10, pp. 1161–1181, Oct. 1996, doi: 10.1097/00000478-199610000-00001.

[161] J. A. Winter *et al.*, 'A Role for the Vacuolating Cytotoxin, VacA, in Colonization and Helicobacter pylori-Induced Metaplasia in the Stomach', 2014, doi: 10.1093/infdis/jiu154.

[162] L. Lang and F. García, 'Comparison of E-test and disk diffusion assay to evaluate resistance of Helicobacter pylori isolates to amoxicillin, clarithromycin, metronidazole and tetracycline in Costa Rica', *Int J Antimicrob Agents*, vol. 24, no. 6, pp. 572–577, Dec. 2004, doi: 10.1016/J.IJANTIMICAG.2004.07.009.

[163] J. Alarcón-Millán *et al.*, 'Clarithromycin resistance and prevalence of Helicobacter pylori virulent genotypes in patients from Southern México with chronic gastritis', *Infection, Genetics and Evolution*, vol. 44, pp. 190–198, 2016, doi: 10.1016/j.meegid.2016.06.044.

[164] E. Glocker, C. Bogdan, and M. Kist, 'Characterization of rifampicin-resistant clinical Helicobacter pylori isolates from Germany', *J Antimicrob Chemother*, vol. 59, no. 5, pp. 874–879, May 2007, doi: 10.1093/JAC/DKM039.

[165] C. Yu *et al.*, 'Levofloxacin Susceptibility Testing for Helicobacter pylori in China: Comparison of E-Test and Disk Diffusion Method', *Helicobacter*, vol. 16, no. 2, pp. 119–123, Apr. 2011, doi: 10.1111/J.1523-5378.2011.00820.X.

[166] S. Chaves, M. Gadanho, R. Tenreiro, and J. Cabrita, 'Assessment of metronidazole susceptibility in Helicobacter pylori: statistical validation and error rate analysis of breakpoints determined by the disk diffusion test.', *J Clin Microbiol*, vol. 37, no. 5, pp. 1628–31, May 1999, Accessed: Jun. 26, 2020. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/10203543

[167] EUCAST, 'EUCAST clinical breakpoints', https://eucast.org/clinical_breakpoints/.

[168] A. K. Walduck and S. Raghavan, 'Immunity and Vaccine Development Against Helicobacter pylori', *Adv Exp Med Biol*, vol. 1149, pp. 257–275, 2019, doi: 10.1007/5584_2019_370.

[169] G. Ayala, W. I. Escobedo-Hinojosa, C. F. de La Cruz-Herrera, and I. Romero, 'Exploring alternative treatments for Helicobacter pylori infection', *World Journal of Gastroenterology : WJG*, vol. 20, no. 6, p. 1450, Feb. 2014, doi: 10.3748/WJG.V20.I6.1450.

[170] C. McNulty, 'Test and treat for Helicobacter pylori (HP) in Dyspepsia: Quick Reference Guide for Primary Care', 2017.

[171] C. A. Fallone, S. F. Moss, and P. Malfertheiner, 'Reconciliation of Recent Helicobacter pylori Treatment Guidelines in a Time of Increasing Resistance to Antibiotics', *Gastroenterology*, vol.

157, no. 1, pp. 44–53, Jul. 2019, doi: 10.1053/J.GASTRO.2019.04.011.

[172] H. K. Jung *et al.*, ‘Evidence based guidelines for the treatment of Helicobacter pylori infection in Korea 2020’, *Korean J Intern Med*, vol. 36, no. 4, pp. 807–838, Jul. 2021, doi: 10.3904/KJIM.2020.701.

[173] P. Malfertheiner *et al.*, ‘Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report.’, *Gut*, vol. 66, no. 1, pp. 6–30, Jan. 2017, doi: 10.1136/gutjnl-2016-312288.

[174] D. Y. Graham, ‘Implications of the paradigm shift in management of Helicobacter pylori infections’, *Therap Adv Gastroenterol*, vol. 16, Jan. 2023, doi: 10.1177/17562848231160858/ASSET/IMAGES/LARGE/10.1177_17562848231160858-FIG7.JPEG.

[175] I. L. P. Beales, ‘Efficacy of Helicobacter pylori eradication therapies: a single centre observational study’, *BMC Gastroenterol*, vol. 1, Aug. 2001, doi: 10.1186/1471-230X-1-7.

[176] J. W. Ogle, ‘Effervescing Bismuth Water’, *Br Med J*, vol. 1, no. 165, pp. 249–250, Feb. 1864, doi: 10.1136/BMJ.1.165.249.

[177] H. Alkim, A. R. Koksal, S. Boga, I. Sen, and C. Alkim, 'Role of Bismuth in the Eradication of Helicobacter pylori', *Am J Ther*, vol. 24, no. 6, pp. e751–e757, 2017, doi: 10.1097/MJT.0000000000000389.

[178] C. S. Goodwin, B. J. Marshall, E. D. Blincow, D. H. Wilson, S. Blackbourn, and M. Phillips, 'Prevention of nitroimidazole resistance in Campylobacter pylori by coadministration of colloidal bismuth subcitrate: clinical and in vitro studies.', *J Clin Pathol*, vol. 41, no. 2, pp. 207–10, Feb. 1988, doi: 10.1136/jcp.41.2.207.

[179] M. P. Dore, H. Lu, and D. Y. Graham, 'Role of bismuth in improving Helicobacter pylori eradication with triple therapy.', *Gut*, vol. 65, no. 5, pp. 870–8, May 2016, doi: 10.1136/gutjnl-2015-311019.

[180] L. Perez Aldana *et al.*, 'The relationship between consumption of antimicrobial agents and the prevalence of primary Helicobacter pylori resistance.', *Helicobacter*, vol. 7, no. 5, pp. 306–9, Oct. 2002, doi: 10.1046/j.1523-5378.2002.00096.x.

[181] R. Ghotaslou, H. E. Leylabadlo, and Y. M. Asl, 'Prevalence of antibiotic resistance in Helicobacter pylori: A recent literature review.', *World J Methodol*, vol. 5, no. 3, pp. 164–74, Sep. 2015, doi: 10.5662/wjm.v5.i3.164.

[182] G. V. Asokan, T. Ramadhan, E. Ahmed, and H. Sanad, 'WHO Global Priority Pathogens List: A Bibliometric Analysis of Medline-PubMed for Knowledge Mobilization to Infection Prevention and Control Practices in Bahrain', *Oman Med J*, vol. 34, no. 3, p. 184, 2019, doi: 10.5001/OMJ.2019.37.

[183] L. Boyanova, P. Hadzhiyski, R. Gergova, and R. Markovska, 'Evolution of Helicobacter pylori Resistance to Antibiotics: A Topic of Increasing Concern', *Antibiotics*, vol. 12, no. 2, Feb. 2023, doi: 10.3390/ANTIBIOTICS12020332.

[184] H. Jaka *et al.*, 'The magnitude of antibiotic resistance to Helicobacter pylori in Africa and identified mutations which confer resistance to antibiotics: Systematic review and meta-analysis', *BMC Infect Dis*, vol. 18, no. 1, pp. 1–10, Apr. 2018, doi: 10.1186/S12879-018-3099-4/TABLES/3.

[185] I. Chopra and M. Roberts, 'Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance', *Microbiology and Molecular Biology Reviews*, vol. 65, no. 2, pp. 232–260, Jun. 2001, doi: 10.1128/mmbr.65.2.232-260.2001.

[186] M. M. Gerrits, M. R. De Zoete, N. L. A. Arents, E. J. Kuipers, and J. G. Kusters, '16S rRNA mutation-mediated tetracycline resistance in Helicobacter pylori', *Antimicrob Agents Chemother*, vol. 46, no. 9, pp. 2996–3000, 2002, doi:

10.1128/AAC.46.9.2996-3000.2002/ASSET/E183945D-156D-433C-9116-60554F82FFF4/ASSETS/GRAPHIC/AC0920148003.JPEG.

[187] A. B. Shrestha *et al.*, 'Drug Resistance Patterns of Commonly Used Antibiotics for the Treatment of Helicobacter pylori Infection among South Asian Countries: A Systematic Review and Meta-Analysis', *Trop Med Infect Dis*, vol. 8, no. 3, p. 172, Mar. 2023, doi: 10.3390/TROPICALMED8030172/S1.

[188] S. Dutta, S. Jain, K. Das, P. Verma, A. Som, and R. Das, 'Primary antibiotic resistance of Helicobacter pylori in India over the past two decades: A systematic review', *Helicobacter*, vol. 29, no. 1, Jan. 2024, doi: 10.1111/HEL.13057.

[189] Y. H. Kwon, *Amoxicillin*. StatPearls Publishing, 2016. doi: 10.1007/978-981-287-706-2_37.

[190] Y. S. Tseng *et al.*, 'Amoxicillin resistance with β-lactamase production in Helicobacter pylori', *Eur J Clin Invest*, vol. 39, no. 9, pp. 807–812, Sep. 2009, doi: 10.1111/J.1365-2362.2009.02166.X.

[191] S. Ansari and Y. Yamaoka, 'Helicobacter pylori Infection, Its Laboratory Diagnosis, and Antimicrobial Resistance: a Perspective of Clinical Relevance', *Clin Microbiol Rev*, vol. 35, no. 3, Sep. 2022, doi: 10.1128/CMR.00258-

21/ASSET/BAB3C181-DB79-4524-AEA6-F6670144F6CC/ASSETS/IMAGES/LARGE/CMR.00258-21-F005.JPG.

[192] T. T. Tran *et al.*, 'Emergence of amoxicillin resistance and identification of novel mutations of the pbp1A gene in Helicobacter pylori in Vietnam', *BMC Microbiol*, vol. 22, no. 1, pp. 1–12, Dec. 2022, doi: 10.1186/S12866-022-02463-8/TABLES/4.

[193] E. Rimbara, N. Noguchi, T. Kawai, and M. Sasatsu, 'Mutations in penicillin-binding proteins 1, 2 and 3 are responsible for amoxicillin resistance in Helicobacter pylori', *Journal of Antimicrobial Chemotherapy*, vol. 61, no. 5, pp. 995–998, May 2008, doi: 10.1093/JAC/DKN051.

[194] R. I. Dascălu *et al.*, 'Multidrug resistance in Helicobacter pylori infection', *Front Microbiol*, vol. 14, 2023, doi: 10.3389/FMICB.2023.1128497.

[195] S. D. Georgopoulos *et al.*, 'Randomized clinical trial comparing ten day concomitant and sequential therapies for Helicobacter pylori eradication in a high clarithromycin resistance area.', *Eur J Intern Med*, vol. 32, pp. 84–90, Jul. 2016, doi: 10.1016/j.ejim.2016.04.011.

[196] C. B. Weir and J. K. Le, *Metronidazole*. StatPearls Publishing, 2020.

[197] Y. Gong *et al.*, 'RdxA Diversity and Mutations Associated with Metronidazole Resistance of Helicobacter pylori', *Microbiol Spectr*, vol. 11, no. 2, Apr. 2023, doi: 10.1128/SPECTRUM.03903-22.

[198] I. Thung *et al.*, 'Review article: the global emergence of Helicobacter pylori antibiotic resistance', *Aliment Pharmacol Ther*, vol. 43, no. 4, pp. 514–533, Feb. 2016, doi: 10.1111/APT.13497.

[199] L.-H. Wang, H. Cheng, F.-L. Hu, and J. Li, 'Distribution of gyrA mutations in fluoroquinolone-resistant Helicobacter pylori strains.', *World J Gastroenterol*, vol. 16, no. 18, pp. 2272–7, May 2010, doi: 10.3748/wjg.v16.i18.2272.

[200] J. M. Blondeau, 'Fluoroquinolones: mechanism of action, classification, and development of resistance', *Surv Ophthalmol*, vol. 49, no. 2, pp. S73–S78, Mar. 2004, doi: 10.1016/J.SURVOPHTHAL.2004.01.005.

[201] T. Ziver-Sarp *et al.*, 'Point mutations at gyrA and gyrB genes of levofloxacin resistant helicobacter pylori strains and dual resistance with clarithromycin', *Clin Lab*, vol. 67, no. 10, p. 2369, Oct. 2021, doi: 10.7754/CLIN.LAB.2021.210843.

[202] S. A. Chisholm, E. L. Teare, K. Davies, and R. J. Owen, 'Surveillance of primary antibiotic resistance of Helicobacter pylori at centres in England and Wales over a six-year period

(2000-2005).', *Euro Surveill*, vol. 12, no. 7, pp. E3-4, Jul. 2007, doi: 10.2807/esm.12.07.00721-en.

[203] T. Nishizawa and H. Suzuki, 'Mechanisms of Helicobacter pylori antibiotic resistance and molecular testing', *Front Mol Biosci*, vol. 1, no. OCT, p. 116357, Oct. 2014, doi: 10.3389/FMOLB.2014.00019/BIBTEX.

[204] T. J. Borody *et al.*, 'Efficacy and safety of rifabutin-containing "rescue therapy" for resistant Helicobacter pylori infection', *Aliment Pharmacol Ther*, vol. 23, no. 4, pp. 481–488, Feb. 2006, doi: 10.1111/J.1365-2036.2006.02793.X.

[205] A. V. Borraccino *et al.*, 'Rifabutin as salvage therapy for Helicobacter pylori eradication: Cornerstones and novelties', *World J Gastroenterol*, vol. 28, no. 45, p. 6356, Dec. 2022, doi: 10.3748/WJG.V28.I45.6356.

[206] D. Van Der Poorten and P. H. Katelaris, 'The effectiveness of rifabutin triple therapy for patients with difficult-to-eradicate Helicobacter pylori in clinical practice', *Aliment Pharmacol Ther*, vol. 26, no. 11–12, pp. 1537–1542, Dec. 2007, doi: 10.1111/J.1365-2036.2007.03531.X.

[207] C. J. Kuo *et al.*, 'Rescue therapy with rifabutin regimen for refractory Helicobacter pylori infection with dual drug-resistant strains', *BMC Gastroenterol*, vol. 20, no. 1, pp. 1–5, Jul. 2020, doi: 10.1186/S12876-020-01370-4/TABLES/1.

[208] S. Suzuki *et al.*, ‘Past rifampicin dosing determines rifabutin resistance of Helicobacter pylori’, *Digestion*, vol. 79, no. 1, pp. 1–4, Mar. 2009, doi: 10.1159/000191204.

[209] T. Yang *et al.*, ‘The Inappropriateness of Using Rifampicin E-Test to Predict Rifabutin Resistance in Helicobacter pylori’, *J Infect Dis*, vol. 226, no. Supplement_5, pp. S479–S485, Dec. 2022, doi: 10.1093/INFDIS/JIAC417.

[210] ‘Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022’, 2022, Accessed: May 10, 2024. [Online]. Available: https://www.who.int/publications/book-orders.

[211] A. O’Connor *et al.*, ‘Helicobacter pylori resistance rates for levofloxacin, tetracycline and rifabutin among Irish isolates at a reference centre’, *Ir J Med Sci*, vol. 182, no. 4, pp. 693–695, Dec. 2013, doi: 10.1007/S11845-013-0957-3/TABLES/1.

[212] E. A. Campbell *et al.*, ‘Structural mechanism for rifampicin inhibition of bacterial rna polymerase.’, *Cell*, vol. 104, no. 6, pp. 901–12, Mar. 2001, doi: 10.1016/s0092-8674(01)00286-0.

[213] M. Heep, D. Beck, E. Bayerdörffer, and N. Lehn, ‘Rifampin and rifabutin resistance mechanism in Helicobacter pylori’, 1999. doi: 10.1128/aac.43.6.1497.

[214] E. A. B. Cameron, K. U. Powell, L. Baldwin, P. Jones, G. D. Bell, and S. G. J. Williams, 'Helicobacter pylori: antibiotic resistance and eradication rates in Suffolk, UK, 1991–2001', *J Med Microbiol*, vol. 53, no. 6, pp. 535–538, Jun. 2004, doi: 10.1099/jmm.0.05499-0.

[215] H. K. Parsons, M. J. Carter, D. S. Sanders, T. Winstanley, and A. J. Lobo, 'Helicobacter pylori antimicrobial resistance in the United Kingdom: the effect of age, sex and socio-economic status', *Aliment Pharmacol Ther*, vol. 15, no. 9, pp. 1473–1478, Sep. 2001, doi: 10.1046/j.1365-2036.2001.01068.x.

[216] N. Banatvala *et al.*, 'High prevalence of Helicobacter pylori metronidazole resistance in migrants to east London: relation with previous nitroimidazole exposure and gastroduodenal disease.', *Gut*, vol. 35, no. 11, pp. 1562–6, Nov. 1994, doi: 10.1136/gut.35.11.1562.

[217] Y. Glupczynski, F. Mégraud, M. Lopez-Brea, and L. Andersen, 'European Multicentre Survey of in Vitro Antimicrobial Resistance in Helicobacter pylori', *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 20, no. 11, pp. 820–823, Nov. 2001, doi: 10.1007/s100960100611.

[218] N. C. Elviss, R. J. Owen, A. Breathnach, C. Palmer, and N. Shetty, 'Helicobacter pylori antibiotic-resistance patterns and risk factors in adult dyspeptic patients from ethnically diverse

populations in central and south London during 2000’, *J Med Microbiol*, vol. 54, no. 6, pp. 567–574, Jun. 2005, doi: 10.1099/jmm.0.45896-0.

[219] N. C. Elviss, R. J. Owen, J. Xerry, A. M. Walker, and K. Davies, ‘Helicobacter pylori antibiotic resistance patterns and genotypes in adult dyspeptic patients from a regional population in North Wales’, *Journal of Antimicrobial Chemotherapy*, vol. 54, no. 2, pp. 435–440, Jul. 2004, doi: 10.1093/jac/dkh343.

[220] C. A. M. McNulty *et al.*, ‘Is Helicobacter pylori antibiotic resistance surveillance needed and how can it be delivered?’, *Aliment Pharmacol Ther*, vol. 35, no. 10, pp. 1221–1230, May 2012, doi: 10.1111/j.1365-2036.2012.05083.x.

[221] S. A. Chisholm and R. J. Owen, ‘Frequency and molecular characteristics of ciprofloxacin- and rifampicin-resistant Helicobacter pylori from gastric infections in the UK’, *J Med Microbiol*, vol. 58, no. 10, pp. 1322–1328, Oct. 2009, doi: 10.1099/jmm.0.011270-0.

[222] C. K. Tai, A. Jepson, and L. Marelli, ‘PTU-071 Helicobacter pylori brexit: NICE vs maastricht A comparison of eradication guidelines and resistance in London’, p. A153.1-A153, 2019, doi: 10.1136/gutjnl-2019-bsgabstracts.287.

[223] I. Koumoutsos, J. Klein, E. Compot, S. Hwang, A. Finze, and G. Tritto, 'PTU-062 "The only good H. pylori is a dead H. pylori" – challenges in isolation and eradication', in *Posters*, BMJ Publishing Group Ltd and British Society of Gastroenterology, Jun. 2019, p. A148.1-A148. doi: 10.1136/gutjnl-2019-BSGAbstracts.278.

[224] S. L. Cudmore, K. L. Delgaty, S. F. Hayward-McClelland, D. P. Petrin, and G. E. Garber, 'Treatment of infections caused by metronidazole-resistant Trichomonas vaginalis.', *Clin Microbiol Rev*, vol. 17, no. 4, pp. 783–93, table of contents, Oct. 2004, doi: 10.1128/CMR.17.4.783-793.2004.

[225] S. Puttaswamy, S. K. Gupta, H. Regunath, L. P. Smith, and S. Sengupta, 'A Comprehensive Review of the Present and Future Antibiotic Susceptibility Testing (AST) Systems', *Arch Clin Microbiol*, no. 9, p. 83, 2018, doi: 10.4172/1989-8436.100083.

[226] Z. A. Khan, M. F. Siddiqui, and S. Park, 'Current and Emerging Methods of Antibiotic Susceptibility Testing', *Diagnostics 2019, Vol. 9, Page 49*, vol. 9, no. 2, p. 49, May 2019, doi: 10.3390/DIAGNOSTICS9020049.

[227] K. Syal *et al.*, 'Current and emerging techniques for antibiotic susceptibility tests', *Theranostics*, vol. 7, no. 7, p. 1795, 2017, doi: 10.7150/THNO.19217.

[228] I. Wiegand, K. Hilpert, and R. E. W. Hancock, 'Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances', *Nature Protocols 2008 3:2*, vol. 3, no. 2, pp. 163–175, Jan. 2008, doi: 10.1038/nprot.2007.521.

[229] S. K. Ogata, A. C. Gales, and E. Kawakami, 'Antimicrobial susceptibility testing for Helicobacter pylori isolates from Brazilian children and adolescents: comparing agar dilution, E-test, and disk diffusion', *Brazilian Journal of Microbiology*, vol. 45, no. 4, pp. 1439–1448, 2014, doi: 10.1590/S1517-83822014000400039.

[230] M. Miftahussurur *et al.*, 'E-test versus agar dilution for antibiotic susceptibility testing of Helicobacter pylori: A comparison study', *BMC Res Notes*, vol. 13, no. 1, pp. 1–6, Jan. 2020, doi: 10.1186/S13104-019-4877-9/FIGURES/2.

[231] L. Mahmoudi, F. Sharifzadeh, S. Mousavi, B. Pourabbas, and R. Niknam, 'Susceptibility testing of Helicobacter pylori: Comparison of E-test and Disk Diffusion for Metronidazole and Mutations in rdxA gene sequences of Helicobacter pylori strains', *Trends in Pharmaceutical Sciences*, vol. 1, no. 4, pp. 235–242, Dec. 2015, Accessed: Jun. 06, 2024. [Online]. Available: https://tips.sums.ac.ir/article_42175.html

[232] ‘eucast: Previous versions of documents’. Accessed: Jun. 06, 2024. [Online]. Available: https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents

[233] H. Li *et al.*, ‘Need for standardization and harmonization of Helicobacter pylori antimicrobial susceptibility testing’, *Helicobacter*, vol. 27, no. 2, Apr. 2022, doi: 10.1111/HEL.12873.

[234] T. P. Cusack *et al.*, ‘Time to switch from CLSI to EUCAST? A Southeast Asian perspective’, *Clinical Microbiology and Infection*, vol. 25, no. 7, p. 782, Jul. 2019, doi: 10.1016/J.CMI.2019.03.016.

[235] ‘eucast: EUCAST setting breakpoints.’ Accessed: Jun. 05, 2024. [Online]. Available: https://www.eucast.org/clinical_breakpoints_and_dosing/eucast_setting_breakpoints

[236] M. J. Chen *et al.*, ‘Impact of amoxicillin resistance on the efficacy of amoxicillin-containing regimens for Helicobacter pylori eradication: analysis of five randomized trials’, *Journal of Antimicrobial Chemotherapy*, vol. 72, no. 12, pp. 3481–3489, Dec. 2017, doi: 10.1093/JAC/DKX320.

[237] J. P. Schubert *et al.*, ‘Geospatial analysis of Helicobacter pylori infection in South Australia: Should location influence

eradication therapy?', *J Gastroenterol Hepatol*, vol. 37, no. 7, pp. 1263–1274, Jul. 2022, doi: 10.1111/JGH.15832.

[238] L. Wang *et al.*, 'Dynamic changes in antibiotic resistance genes and gut microbiota after Helicobacter pylori eradication therapies', *Helicobacter*, vol. 27, no. 2, p. e12871, Apr. 2022, doi: 10.1111/HEL.12871.

[239] O. Sjomina *et al.*, 'Clarithromycin-containing triple therapy for Helicobacter pylori eradication is inducing increased long-term resistant bacteria communities in the gut', *Gut*, 2023, doi: 10.1136/GUTJNL-2023-329792.

[240] UK Health Security Agency, 'English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2021 to 2022'. Accessed: Jun. 02, 2024. [Online]. Available: https://webarchive.nationalarchives.gov.uk/ukgwa/20231002172235/https://www.gov.uk/government/publications/english-surveillance-programme-antimicrobial-utilisation-and-resistance-espaur-report

[241] UK Health Security Agency, 'English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2022 to 2023', 2023.

[242] M. H. Thornhill, M. J. Dayer, M. J. Durkin, P. B. Lockhart, and L. M. Baddour, 'Oral antibiotic prescribing by NHS dentists in

England 2010–2017', *Br Dent J*, vol. 227, no. 12, p. 1044, Dec. 2019, doi: 10.1038/S41415-019-1002-3.

[243] L. Yu *et al.*, 'High-dose PPI-amoxicillin dual therapy with or without bismuth for first-line Helicobacter pylori therapy: A randomized trial', *Helicobacter*, p. e12596, May 2019, doi: 10.1111/hel.12596.

[244] Y. Ji and H. Lu, 'Meta-analysis: High-dose vs. low-dose metronidazole-containing therapies for Helicobacter pylori eradication treatment', *PLoS One*, vol. 13, no. 1, Jan. 2018, doi: 10.1371/JOURNAL.PONE.0189888.

[245] F. Megraud *et al.*, 'Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption', *Gut*, vol. 62, no. 1, pp. 34–42, Jan. 2013, doi: 10.1136/GUTJNL-2012-302254.

[246] F. Mégraud *et al.*, 'Survey of the antimicrobial resistance of Helicobacter pylori in France in 2018 and evolution during the previous 5 years', *Helicobacter*, vol. 26, no. 1, p. e12767, Feb. 2021, doi: 10.1111/HEL.12767.

[247] A. O'Connor *et al.*, 'Helicobacter pylori resistance to metronidazole and clarithromycin in Ireland', *Eur J Gastroenterol Hepatol*, vol. 22, no. 9, pp. 1123–1127, Sep. 2010, doi: 10.1097/MEG.0B013E328338E43D.

[248] F. Mégraud, ‘H pylori antibiotic resistance: prevalence, importance, and advances in testing’, *Gut*, vol. 53, no. 9, pp. 1374–1384, Sep. 2004, doi: 10.1136/GUT.2003.022111.

[249] J. M. C. Gautron, G. T. Thanh, V. Barasa, and G. Voltolina, ‘Using intersectionality to study gender and antimicrobial resistance in low- and middle-income countries’, *Health Policy Plan*, vol. 38, no. 9, p. 1017, Nov. 2023, doi: 10.1093/HEAPOL/CZAD054.

[250] M. B. Steinberg, A. Akincigil, E. J. Kim, R. Shallis, and C. D. Delnevo, ‘Tobacco Smoking as a Risk Factor for Increased Antibiotic Prescription’, *Am J Prev Med*, vol. 50, no. 6, pp. 692–698, Jun. 2016, doi: 10.1016/J.AMEPRE.2015.11.009.

[251] J. Yu, P. Yang, X. Qin, C. Li, Y. Lv, and X. Wang, ‘Impact of smoking on the eradication of Helicobacter pylori’, *Helicobacter*, vol. 27, no. 1, Feb. 2022, doi: 10.1111/HEL.12860.

[252] T. Suzuki *et al.*, ‘Smoking increases the treatment failure for Helicobacter pylori eradication’, *American Journal of Medicine*, vol. 119, no. 3, pp. 217–224, Mar. 2006, doi: 10.1016/j.amjmed.2005.10.003.

[253] G. Losurdo *et al.*, ‘Helicobacter pylori Secondary Antibiotic Resistance after One or More Eradication Failure: A Genotypic

Stool Analysis Study', *Antibiotics*, vol. 13, no. 4, Apr. 2024, doi: 10.3390/ANTIBIOTICS13040336.

[254] J. Yang *et al.*, 'Antimicrobial Resistance of Helicobacter Pylori Among Low-resource Chinese Minorities.', *Altern Ther Health Med*, 2022.

[255] F. Ailloud *et al.*, 'Within-host evolution of Helicobacter pylori shaped by niche-specific adaptation, intragastric migrations and selective sweeps', *Nature Communications 2019 10:1*, vol. 10, no. 1, pp. 1–13, May 2019, doi: 10.1038/s41467-019-10050-1.

[256] R. M. Rolon, S. A. Cunningham, J. N. Mandrekar, E. T. Polo, and R. Patel, 'Clinical Evaluation of a Real-Time PCR Assay for Simultaneous Detection of Helicobacter pylori and Genotypic Markers of Clarithromycin Resistance Directly from Stool', *J Clin Microbiol*, vol. 59, no. 5, May 2021, doi: 10.1128/JCM.03040-20.

[257] M. Pichon *et al.*, 'Diagnostic Accuracy of a Noninvasive Test for Detection of Helicobacter pylori and Resistance to Clarithromycin in Stool by the Amplidiag H. pylori+ClariR Real-Time PCR Assay', *J Clin Microbiol*, vol. 58, no. 4, 2020, doi: 10.1128/JCM.01787-19.

[258] 'eucast: Disk diffusion methodology', 2023. Accessed: Jun. 19, 2024. [Online]. Available:

https://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology

[259] J. V. John *et al.*, 'Nanofiber capsules for minimally invasive sampling of biological specimens from gastrointestinal tract', *Acta Biomater*, vol. 146, pp. 211–221, Jul. 2022, doi: 10.1016/J.ACTBIO.2022.04.045.

[260] Y. Li, H. Choi, K. Leung, F. Jiang, D. Y. Graham, and W. K. Leung, 'Global prevalence of Helicobacter pylori infection between 1980 and 2022: a systematic review and meta-analysis', *Lancet Gastroenterol Hepatol*, vol. 8, no. 6, pp. 553–564, Jun. 2023, doi: 10.1016/S2468-1253(23)00070-5.

[261] J. J. C. Ho, M. Navarro, K. Sawyer, Y. Elfanagely, and S. F. Moss, 'Helicobacter pylori Antibiotic Resistance in the United States Between 2011 and 2021: A Systematic Review and Meta-Analysis', *Am J Gastroenterol*, vol. 117, no. 8, pp. 1221–1230, Aug. 2022, doi: 10.14309/AJG.0000000000001828.

[262] 'Fluoroquinolone antibiotics: new restrictions and precautions for use due to very rare reports of disabling and potentially long-lasting or irreversible side effects - GOV.UK'. Accessed: Oct. 22, 2024. [Online]. Available: https://www.gov.uk/drug-safety-update/fluoroquinolone-antibiotics-new-restrictions-and-precautions-for-use-due-to-very-rare-reports-of-

disabling-and-potentially-long-lasting-or-irreversible-side-effects

[263] M. Keikha, P. Askari, K. Ghazvini, and M. Karbalaei, 'Levofloxacin-based therapy as an efficient alternative for eradicating Helicobacter pylori infection in Iran: a systematic review and meta-analysis', *J Glob Antimicrob Resist*, vol. 29, pp. 420–429, Jun. 2022, doi: 10.1016/J.JGAR.2021.10.019.

[264] D. Pohl, P. M. Keller, V. Bordier, and K. Wagner, 'Review of current diagnostic methods and advances in Helicobacter pylori diagnostics in the era of next generation sequencing.', *World J Gastroenterol*, vol. 25, no. 32, pp. 4629–4660, Aug. 2019, doi: 10.3748/wjg.v25.i32.4629.

[265] S. F. Moss, L. P. Dang, D. Chua, J. Sobrado, Y. Zhou, and D. Y. Graham, 'Comparable Results of Helicobacter pylori Antibiotic Resistance Testing of Stools vs Gastric Biopsies Using Next-Generation Sequencing', *Gastroenterology*, vol. 162, no. 7, pp. 2095-2097.e2, Jun. 2022, doi: 10.1053/j.gastro.2022.02.027.

[266] S. Bonilla, J. Goldsmith, P. Mitchell, and A. Bousvaros, 'Helicobacter pylori Antimicrobial Resistance Using Next-Generation Sequencing in Stool Samples in a Pediatric Population', *J Pediatr Gastroenterol Nutr*, vol. 77, no. 5, pp. 623–627, Nov. 2023, doi: 10.1097/MPG.0000000000003908.

[267] C. M. Oliphant, G. M. Green, K. Permanente, and R. W. Sloan, ‘Quinolones: A Comprehensive Review’, *Am Fam Physician*, vol. 65, no. 3, pp. 455–465, Feb. 2002, Accessed: Jun. 24, 2024. [Online]. Available: https://www.aafp.org/pubs/afp/issues/2002/0201/p455.html

[268] ‘WHO Model Lists of Essential Medicines’. Accessed: Jul. 03, 2024. [Online]. Available: https://www.who.int/groups/expert-committee-on-selection-and-use-of-essential-medicines/essential-medicines-lists

[269] ‘Levofloxacin: some indications restricted - GOV.UK’. Accessed: Jul. 03, 2024. [Online]. Available: https://www.gov.uk/drug-safety-update/levofloxacin-some-indications-restricted

[270] ‘Levofloxacin: Uses, Interactions, Mechanism of Action | DrugBank Online’. Accessed: Jul. 03, 2024. [Online]. Available: https://go.drugbank.com/drugs/DB01137

[271] H.-Y. Gan *et al.*, ‘Efficacy of two different dosages of levofloxacin in curing Helicobacter pylori infection: A Prospective, Single-Center, randomized clinical trial’, *Sci Rep*, vol. 8, no. 1, p. 9045, Dec. 2018, doi: 10.1038/s41598-018-27482-2.

[272] D. N. Fish and A. T. Chow, ‘The Clinical Pharmacokinetics of Levofloxacin’, *Clin Pharmacokinet*, vol. 32, no. 2, pp. 101–119, Feb. 1997, doi: 10.2165/00003088-199732020-00002.

[273] G. K. Kim, ‘The Risk of Fluoroquinolone-induced Tendinopathy and Tendon Rupture: What Does The Clinician Need To Know?’, *J Clin Aesthet Dermatol*, vol. 3, no. 4, pp. 49–54, Apr. 2010.

[274] K. Akahane, Y. Tsutomi, Y. Kimura, and Y. Kitano, ‘Levofloxacin, an optical isomer of ofloxacin, has attenuated epileptogenic activity in mice and inhibitory potency in GABA receptor binding.’, *Chemotherapy*, vol. 40, no. 6, pp. 412–7, 1994, doi: 10.1159/000239301.

[275] L. Takser and R. Grad, ‘Acute psychotic symptoms following a single dose of levofloxacin.’, *Clin Case Rep*, vol. 5, no. 12, pp. 2136–2137, 2017, doi: 10.1002/ccr3.1240.

[276] ‘FDA Drug Safety Communication: FDA updates warnings for oral and injectable fluoroquinolone antibiotics due to disabling side effects | FDA’. Accessed: Jun. 24, 2024. [Online]. Available: https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-updates-warnings-oral-and-injectable-fluoroquinolone-antibiotics

[277] H. H. Liu, ‘Safety profile of the fluoroquinolones: Focus on levofloxacin’, *Drug Saf*, vol. 33, no. 5, pp. 353–369, Nov.

2010, doi: 10.2165/11536360-000000000-00000/FIGURES/TAB1.

[278] F. Collin, S. Karkare, and A. Maxwell, ‘Exploiting bacterial DNA gyrase as a drug target: current state and perspectives’, *Applied Microbiology and Biotechnology 2011 92:3*, vol. 92, no. 3, pp. 479–497, Sep. 2011, doi: 10.1007/S00253-011-3557-Z.

[279] S. C. Kampranis, A. D. Bates, and A. Maxwell, ‘A model for the mechanism of strand passage by DNA gyrase’, *Proc Natl Acad Sci U S A*, vol. 96, no. 15, p. 8414, Jul. 1999, doi: 10.1073/PNAS.96.15.8414.

[280] M. K. Wall, L. A. Mitchena, and A. Maxwell, ‘Arabidopsis thaliana DNA gyrase is targeted to chloroplasts and mitochondria’, *Proc Natl Acad Sci U S A*, vol. 101, no. 20, pp. 7821–7826, May 2004, doi: 10.1073/PNAS.0400836101/SUPPL_FILE/00836SUPPTEXT.HTML.

[281] R. Spagnuolo, G. G. M. Scarlata, M. R. Paravati, L. Abenavoli, and F. Luzza, ‘Change in Diagnosis of Helicobacter pylori Infection in the Treatment-Failure Era’, *Antibiotics 2024, Vol. 13, Page 357*, vol. 13, no. 4, p. 357, Apr. 2024, doi: 10.3390/ANTIBIOTICS13040357.

[282] A. C. Spencer and S. S. Panda, 'DNA Gyrase as a Target for Quinolones', *Biomedicines*, vol. 11, no. 2, Feb. 2023, doi: 10.3390/BIOMEDICINES11020371.

[283] W. Goedecke, 'Topoisomerases', *xPharm: The Comprehensive Pharmacology Reference*, pp. 1–2, 2007, doi: 10.1016/B978-008055232-3.60609-9.

[284] A. Wohlkonig *et al.*, 'Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance', *Nature Structural & Molecular Biology 2010 17:9*, vol. 17, no. 9, pp. 1152–1153, Aug. 2010, doi: 10.1038/nsmb.1892.

[285] L. F. Liu, C. C. Liu, and B. M. Alberts, 'Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break', *Cell*, vol. 19, no. 3, pp. 697–707, 1980, doi: 10.1016/S0092-8674(80)80046-8.

[286] A. Fàbrega, S. Madurga, E. Giralt, and J. Vila, 'Mechanism of action of and resistance to quinolones', *Microb Biotechnol*, vol. 2, no. 1, pp. 40–61, Jan. 2009, doi: 10.1111/j.1751-7915.2008.00063.x.

[287] S. Madurga, J. Sánchez-Céspedes, I. Belda, J. Vila, and E. Giralt, 'Mechanism of binding of fluoroquinolones to the quinolone resistance-determining region of DNA gyrase: Towards an understanding of the molecular basis of quinolone

resistance', *ChemBioChem*, vol. 9, no. 13, pp. 2081–2086, Sep. 2008, doi: 10.1002/CBIC.200800041.

[288] Y. Elshenawi, S. Hu, and S. Hathroubi, 'Biofilm of Helicobacter pylori: Life Cycle, Features, and Treatment Options', *Antibiotics*, vol. 12, no. 8, Aug. 2023, doi: 10.3390/ANTIBIOTICS12081260.

[289] Z. Zhang, Z. Q. Liu, P. Y. Zheng, F. A. Tang, and P. C. Yang, 'Influence of efflux pump inhibitors on the multidrug resistance of Helicobacter pylori', *World Journal of Gastroenterology : WJG*, vol. 16, no. 10, p. 1279, Mar. 2010, doi: 10.3748/WJG.V16.I10.1279.

[290] D. S. Raj, D. K. Kesavan, N. Muthusamy, and S. Umamaheswari, 'Efflux pumps potential drug targets to circumvent drug Resistance – Multi drug efflux pumps of Helicobacter pylori', *Mater Today Proc*, vol. 45, pp. 2976–2981, Jan. 2021, doi: 10.1016/J.MATPR.2020.11.955.

[291] M. M. Gerrits, A. H. M. Van Vliet, E. J. Kuipers, and J. G. Kusters, 'Helicobacter pylori and antimicrobial resistance: molecular mechanisms and clinical implications', 2006, Accessed: Jul. 08, 2024. [Online]. Available: http://infection.thelancet.comVol

[292] E. Rimbara, N. Noguchi, T. Kawai, and M. Sasatsu, 'Fluoroquinolone Resistance in Helicobacter pylori: Role of

Mutations at Position 87 and 91 of GyrA on the Level of Resistance and Identification of a Resistance Conferring Mutation in GyrB', *Helicobacter*, vol. 17, no. 1, pp. 36–42, Feb. 2012, doi: 10.1111/J.1523-5378.2011.00912.X.

[293] H. Miyachi *et al.*, 'Primary Levofloxacin Resistance and gyrA/B Mutations Among Helicobacter pylori in Japan', *Helicobacter*, vol. 11, no. 4, pp. 243–249, Aug. 2006, doi: 10.1111/j.1523-5378.2006.00415.x.

[294] J. Matsuzaki, H. Suzuki, H. Tsugawa, T. Nishizawa, and T. Hibi, 'Homology model of the DNA gyrase enzyme of Helicobacter pylori, a target of quinolone-based eradication therapy', *J Gastroenterol Hepatol*, vol. 25, no. SUPPL. 1, pp. S7–S10, 2010, doi: 10.1111/J.1440-1746.2010.06245.X.

[295] F. Haumaier, A. Schneider-Fuchs, S. Backert, M. Vieth, W. Sterlacci, and B. M. Wöhrl, 'Rapid Detection of Quinolone Resistance Mutations in gyrA of Helicobacter pylori by Real-Time PCR', *Pathogens*, vol. 11, no. 1, Jan. 2022, doi: 10.3390/PATHOGENS11010059/S1.

[296] M. A. Lanz and D. Klostermeier, 'The GyrA-box determines the geometry of DNA bound to gyrase and couples DNA binding to the nucleotide cycle', *Nucleic Acids Res*, vol. 40, no. 21, pp. 10893–10903, Nov. 2012, doi: 10.1093/NAR/GKS852.

[297] N. Salehi *et al.*, ‘New insights into resistance of Helicobacter pylori against third- and fourth-generation fluoroquinolones: A molecular docking study of prevalent GyrA mutations’, *Helicobacter*, vol. 24, no. 5, Oct. 2019, doi: 10.1111/HEL.12628.

[298] D. Binyamin, N. Pastukh, A. On, M. Paritsky, and A. Peretz, ‘Phenotypic and genotypic correlation as expressed in Helicobacter pylori resistance to clarithromycin and fluoroquinolones’, *Gut Pathog*, vol. 9, no. 1, pp. 1–8, Aug. 2017, doi: 10.1186/S13099-017-0198-5/TABLES/4.

[299] T. Nishizawa *et al.*, ‘Gatifloxacin Resistance and Mutations in gyrA after Unsuccessful Helicobacter pylori Eradication in Japan’, *Antimicrob Agents Chemother*, vol. 50, no. 4, p. 1538, Apr. 2006, doi: 10.1128/AAC.50.4.1538-1540.2006.

[300] V. P. Tuan *et al.*, ‘A Next-Generation Sequencing-Based Approach to Identify Genetic Determinants of Antibiotic Resistance in Cambodian Helicobacter pylori Clinical Isolates’, *J Clin Med*, vol. 8, no. 6, p. 858, Jun. 2019, doi: 10.3390/JCM8060858.

[301] S. Y. Rhie, J. Y. Park, T. S. Shin, J. W. Kim, B. J. Kim, and J. G. Kim, ‘Discovery of a Novel Mutation in DNA Gyrase and Changes in the Fluoroquinolone Resistance of Helicobacter pylori over a 14-Year Period: A Single Center Study in Korea’,

Antibiotics 2020, Vol. 9, Page 287, vol. 9, no. 6, p. 287, May 2020, doi: 10.3390/ANTIBIOTICS9060287.

[302] H. Mori, H. Suzuki, J. Matsuzaki, T. Masaoka, and T. Kanai, 'Acquisition of double mutation in gyrA caused high resistance to sitafloxacin in Helicobacter pylori after unsuccessful eradication with sitafloxacin-containing regimens', *United European Gastroenterol J*, vol. 6, no. 3, pp. 391–397, Apr. 2018, doi: 10.1177/2050640617737215/SUPPL_FILE/SUPPLEMENTARY_TABLE-1_UEGJ_GYRA_MORI.PDF.

[303] A. Bińkowska, M. M. Biernat, L. Laczmański, and G. Gościniak, 'Molecular Patterns of Resistance Among Helicobacter pylori Strains in South-Western Poland', *Front Microbiol*, vol. 9, p. 410371, Mar. 2018, doi: 10.3389/FMICB.2018.03154/BIBTEX.

[304] J. W. Lee *et al.*, 'Mutations of Helicobacter pylori Associated with Fluoroquinolone Resistance in Korea', *Helicobacter*, vol. 16, no. 4, pp. 301–310, Aug. 2011, doi: 10.1111/J.1523-5378.2011.00840.X.

[305] S. Ferreira, D. R. Correia, M. Oleastro, and F. C. Domingues, 'Arcobacter butzleri Ciprofloxacin Resistance: Point Mutations in DNA Gyrase A and Role on Fitness Cost',

https://home.liebertpub.com/mdr, vol. 24, no. 7, pp. 915–922, Sep. 2018, doi: 10.1089/MDR.2017.0295.

[306] J. M. Liou *et al.*, 'Genotypic Resistance in Helicobacter pylori Strains Correlates with Susceptibility Test and Treatment Outcomes after Levofloxacin- and Clarithromycin-Based Therapies', *Antimicrob Agents Chemother*, vol. 55, no. 3, pp. 1123–1129, Dec. 2010, doi: 10.1128/AAC.01131-10.

[307] V. Cattoir *et al.*, 'Update on fluoroquinolone resistance in Helicobacter pylori: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility', *Int J Antimicrob Agents*, vol. 29, no. 4, pp. 389–396, Apr. 2007, doi: 10.1016/J.IJANTIMICAG.2006.11.007.

[308] M. Garcia, J. Raymond, M. Garnier, J. Cremniter, and C. Burucoa, 'Distribution of Spontaneous gyrA Mutations in 97 Fluoroquinolone-Resistant Helicobacter pylori Isolates Collected in France', *Antimicrob Agents Chemother*, vol. 56, no. 1, pp. 550–551, Jan. 2011, doi: 10.1128/AAC.05243-11.

[309] B. G. Nezami, M. Jani, D. Alouani, D. D. Rhoads, and N. Sadria, 'Helicobacter pylori mutations detected by next-generation sequencing in formalin-fixed, paraffin-embedded gastric biopsy specimens are associated with treatment failure', *J Clin Microbiol*, vol. 57, no. 7, 2019, doi:

10.1128/JCM.01834-18/SUPPL_FILE/JCM.01834-18-SD002.XLSX.

[310] K. Murakami *et al.*, 'Sitafloxacin Activity against Helicobacter pylori Isolates, Including Those with gyrA Mutations', *Antimicrob Agents Chemother*, vol. 53, no. 7, p. 3097, Jul. 2009, doi: 10.1128/AAC.01552-08.

[311] G. Wang, T. J. M. Wilson, Q. Jiang, and D. E. Taylor, 'Spontaneous Mutations That Confer Antibiotic Resistance in Helicobacter pylori', *Antimicrob Agents Chemother*, vol. 45, no. 3, p. 727, 2001, doi: 10.1128/AAC.45.3.727-733.2001.

[312] R. A. Moore, B. Beckthold, S. Wong, A. Kureishi, and L. E. Bryan, 'Nucleotide sequence of the gyrA gene and characterization of ciprofloxacin-resistant mutants of Helicobacter pylori', *Antimicrob Agents Chemother*, vol. 39, no. 1, pp. 107–111, Jan. 1995, doi: 10.1128/AAC.39.1.107.

[313] V. Shetty *et al.*, 'High primary resistance to metronidazole and levofloxacin, and a moderate resistance to clarithromycin in Helicobacter pylori isolated from Karnataka patients', *Gut Pathog*, vol. 11, no. 1, pp. 1–8, May 2019, doi: 10.1186/S13099-019-0305-X/TABLES/5.

[314] J. Tankovic, C. Lascols, Q. Sculo, J.-C. Petit, and C.-J. Soussy, 'Single and double mutations in gyrA but not in gyrB are associated with low- and high-level fluoroquinolone resistance

in Helicobacter pylori.', *Antimicrob Agents Chemother*, vol. 47, no. 12, pp. 3942–4, Dec. 2003, doi: 10.1128/aac.47.12.3942-3944.2003.

[315] L. Bujanda *et al.*, 'Antibiotic resistance prevalence and trends in patients infected with helicobacter pylori in the period 2013–2020: Results of the european registry on h. pylori management (hp-eureg)', *Antibiotics*, vol. 10, no. 9, p. 1058, Sep. 2021, doi: 10.3390/ANTIBIOTICS10091058/S1.

[316] K. A. Fauzia *et al.*, 'Mutations Related to Antibiotics Resistance in Helicobacter pylori Clinical Isolates from Bangladesh', *Antibiotics*, vol. 12, no. 2, p. 279, Feb. 2023, doi: 10.3390/ANTIBIOTICS12020279/S1.

[317] A. Chu, D. Wang, Q. Guo, Z. Lv, Y. Yuan, and Y. Gong, 'Molecular detection of H. pylori antibiotic-resistant genes and molecular docking analysis', *FASEB Journal*, vol. 34, no. 1, pp. 610–618, Jan. 2020, doi: 10.1096/FJ.201900774R.

[318] M. López-Gasca, J. Peña, M. A. García-Amado, F. Michelangeli, and M. Contreras, 'Point Mutations at gyrA and gyrB Genes of Levofloxacin-Resistant Helicobacter pylori Isolates in the Esophageal Mucosa from a Venezuelan Population', *Am J Trop Med Hyg*, vol. 98, no. 4, p. 1051, 2018, doi: 10.4269/AJTMH.17-0478.

[319] J. Matsuzaki *et al.*, 'Efficacy of sitafloxacin-based rescue therapy for Helicobacter pylori after failures of first- and second-line therapies', *Antimicrob Agents Chemother*, vol. 56, no. 3, pp. 1643–1645, Mar. 2012, doi: 10.1128/AAC.05941-11/ASSET/A3970D2E-5B3D-4EE2-80A0-A32E382F614C/ASSETS/GRAPHIC/ZAC9991006420001.JPEG.

[320] H. Mori *et al.*, 'Efficacy of 10-day Sitafloxacin-Containing Third-Line Rescue Therapies for Helicobacter pylori Strains Containing the gyrA Mutation', *Helicobacter*, vol. 21, no. 4, pp. 286–294, Aug. 2016, doi: 10.1111/HEL.12286.

[321] E. Beckman *et al.*, 'A novel stool PCR test for helicobacter pylori may predict clarithromycin resistance and eradication of infection at a high rate', *J Clin Microbiol*, vol. 55, no. 8, pp. 2400–2405, Aug. 2017, doi: 10.1128/JCM.00506-17/ASSET/7ECABD71-1467-41DD-AC19-3F6464E5F25C/ASSETS/GRAPHIC/ZJM9990955840002.JPEG.

[322] T. C. Lorenz, 'Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies', *JoVE (Journal of Visualized Experiments)*, vol. 63, no. 63, p. e3998, May 2012, doi: 10.3791/3998.

[323] 'PCR Basics | Thermo Fisher Scientific - UK'. Accessed: Jul. 17, 2024. [Online]. Available: https://www.thermofisher.com/uk/en/home/life-

science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-basics.html

[324] ‘DreamTaq DNA Polymerase (5 U/µL)’. Accessed: Sep. 02, 2024. [Online]. Available: https://www.thermofisher.com/order/catalog/product/EP0701

[325] ‘Phusion® High-Fidelity DNA Polymerase | NEB’. Accessed: Sep. 02, 2024. [Online]. Available: https://www.neb.com/en-gb/products/m0530-phusion-high-fidelity-dna-polymerase#FAQs---Troubleshooting

[326] H. Y. Ng, W. K. Leung, and K. S. Cheung, ‘Antibiotic Resistance, Susceptibility Testing and Stewardship in Helicobacter pylori Infection’, *International Journal of Molecular Sciences 2023, Vol. 24, Page 11708*, vol. 24, no. 14, p. 11708, Jul. 2023, doi: 10.3390/IJMS241411708.

[327] ‘Leibniz Institute DSMZ: Details’. Accessed: Nov. 04, 2024. [Online]. Available: https://www.dsmz.de/collection/catalogue/details/culture/DSM-21031

[328] E. Garvey *et al.*, ‘High incidence of antibiotic resistance amongst isolates of Helicobacter pylori collected in Nottingham, UK, between 2001 and 2018’, *J Med Microbiol*,

vol. 72, no. 12, p. 001776, Nov. 2023, doi: 10.1099/JMM.0.001776/CITE/REFWORKS.

[329] ‘eucast: Clinical breakpoints and dosing of antibiotics’. Accessed: Oct. 24, 2024. [Online]. Available: https://www.eucast.org/clinical_breakpoints

[330] C. L. Schoch *et al.*, ‘NCBI Taxonomy: a comprehensive update on curation, resources and tools’, *Database (Oxford)*, vol. 2020, 2020, doi: 10.1093/DATABASE/BAAA062.

[331] Z. Zhong *et al.*, ‘A retrospective study of the antibiotic-resistant phenotypes and genotypes of Helicobacter pylori strains in China’, *Am J Cancer Res*, vol. 11, no. 10, p. 5027, 2021, Accessed: Oct. 09, 2024. [Online]. Available: /pmc/articles/PMC8569369/

[332] K. Okonechnikov, O. Golosova, M. Fursov, and the U. team, ‘Unipro UGENE: a unified bioinformatics toolkit’, *Bioinformatics*, vol. 28, no. 8, pp. 1166–1167, Apr. 2012, doi: 10.1093/bioinformatics/bts091.

[333] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, and M. J. E. Sternberg, ‘The Phyre2 web portal for protein modeling, prediction and analysis’, *Nature Protocols 2015 10:6*, vol. 10, no. 6, pp. 845–858, May 2015, doi: 10.1038/nprot.2015.053.

[334] E. F. Pettersen *et al.*, ‘UCSF ChimeraX: Structure visualization for researchers, educators, and developers’, *Protein Sci*, vol. 30, no. 1, p. 70, Jan. 2021, doi: 10.1002/PRO.3943.

[335] ‘Guidelines for PCR Optimization with Thermophilic DNA Polymerases | NEB’. Accessed: Nov. 17, 2024. [Online]. Available: https://www.neb.com/en-gb/tools-and-resources/usage-guidelines/guidelines-for-pcr-optimization-with-thermophilic-dna-polymerases

[336] W. Lu *et al.*, ‘Comparative Analysis of the Full Genome of Helicobacter pylori Isolate Sahul64 Identifies Genes of High Divergence’, *J Bacteriol*, vol. 196, no. 5, p. 1073, Mar. 2014, doi: 10.1128/JB.01021-13.

[337] ‘What are the properties of this polymerase (fidelity, product ends, max amplicon, modified base incorporation, etc.)? | NEB’. Accessed: Nov. 18, 2024. [Online]. Available: https://www.neb.com/en-gb/faqs/2023/07/25/what-are-the-properties-of-this-polymerase-fidelity-product-ends-max-amplicon-modified-base-incorporation-etc

[338] A. Bębenek and I. Ziuzia-Graczyk, ‘Fidelity of DNA replication—a matter of proofreading’, *Current Genetics 2018 64:5*, vol. 64, no. 5, pp. 985–996, Mar. 2018, doi: 10.1007/S00294-018-0820-1.

[339] W. Lan *et al.*, ‘Structural investigation into physiological DNA phosphorothioate modification’, *Scientific Reports 2016 6:1*, vol. 6, no. 1, pp. 1–10, May 2016, doi: 10.1038/srep25737.

[340] S. Bubendorfer *et al.*, ‘Genome-wide analysis of chromosomal import patterns after natural transformation of Helicobacter pylori’, *Nat Commun*, vol. 7, p. 11995, Jun. 2016, doi: 10.1038/NCOMMS11995.

[341] A. Seck *et al.*, ‘Primary antibiotic resistance and associated mechanisms in Helicobacter pylori isolates from Senegalese patients’, *Ann Clin Microbiol Antimicrob*, vol. 12, no. 1, pp. 1–5, Jan. 2013, doi: 10.1186/1476-0711-12-3/TABLES/1.

[342] Y. Lin, Y. Shao, J. Yan, and G. Ye, ‘Antibiotic resistance in Helicobacter pylori: From potential biomolecular mechanisms to clinical practice’, *J Clin Lab Anal*, vol. 37, no. 7, p. e24885, Apr. 2023, doi: 10.1002/JCLA.24885.

[343] E. A. Argueta, J. J. C. Ho, Y. Elfanagely, E. D’Agata, and S. F. Moss, ‘Clinical Implication of Drug Resistance for H. pylori Management’, *Antibiotics*, vol. 11, no. 12, p. 1684, Dec. 2022, doi: 10.3390/ANTIBIOTICS11121684.

[344] ‘gyrA - DNA gyrase subunit A - Helicobacter pylori (strain ATCC 700392 / 26695) (Campylobacter pylori) | UniProtKB | UniProt’. Accessed: Nov. 25, 2024. [Online]. Available: https://www.uniprot.org/uniprotkb/P48370/entry

[345] S. Suerbaum and C. Josenhans, ‘Helicobacter pylori evolution and phenotypic diversification in a changing host’, *Nature Reviews Microbiology 2007 5:6*, vol. 5, no. 6, pp. 441–452, Jun. 2007, doi: 10.1038/nrmicro1658.

[346] B. Björkholm, M. Sjölund, P. G. Falk, O. G. Berg, L. Engstrand, and D. I. Andersson, ‘Mutation frequency and biological cost of antibiotic resistance in Helicobacter pylori’, *Proceedings of the National Academy of Sciences*, vol. 98, no. 25, pp. 14607–14612, Dec. 2001, doi: 10.1073/PNAS.241517298.

[347] A. R. Burmeister, ‘Horizontal Gene Transfer’, *Evol Med Public Health*, vol. 2015, no. 1, p. 193, 2015, doi: 10.1093/EMPH/EOV018.

[348] E. Fernandez-Gonzalez and S. Backert, ‘DNA transfer in the gastric pathogen Helicobacter pylori’, *J Gastroenterol*, vol. 49, no. 4, pp. 594–604, Feb. 2014, doi: 10.1007/S00535-014-0938-Y/TABLES/3.

[349] D. Wilkinson *et al.*, ‘Characterization of a Helicobacter pylori strain with high biofilm-forming ability’, *J Med Microbiol*, vol. 72, no. 6, p. 001710, Jun. 2023, doi: 10.1099/JMM.0.001710/CITE/REFWORKS.

[350] C. Corbinais, A. Mathieu, T. Kortulewski, J. P. Radicella, and S. Marsin, ‘Following transforming DNA in Helicobacter pylori

from uptake to expression', *Mol Microbiol*, vol. 101, no. 6, pp. 1039–1053, Sep. 2016, doi: 10.1111/MMI.13440.

[351] M. Winter, A. Buckling, K. Harms, P. J. Johnsen, and M. Vos, 'Antimicrobial resistance acquisition via natural transformation: context is everything', *Curr Opin Microbiol*, vol. 64, pp. 133–138, Dec. 2021, doi: 10.1016/J.MIB.2021.09.009.

[352] Z. Q. Liu, P. Y. Zheng, and P. C. Yang, 'Efflux pump gene hefA of Helicobacter pylori plays an important role in multidrug resistance', *World Journal of Gastroenterology : WJG*, vol. 14, no. 33, p. 5217, Sep. 2008, doi: 10.3748/WJG.14.5217.

[353] K. Hirata *et al.*, 'Contribution of efflux pumps to clarithromycin resistance in Helicobacter pylori', *J Gastroenterol Hepatol*, vol. 25 Suppl 1, no. SUPPL. 1, 2010, doi: 10.1111/J.1440-1746.2009.06220.X.

[354] X. Ge *et al.*, 'Bifunctional Enzyme SpoT Is Involved in Biofilm Formation of Helicobacter pylori with Multidrug Resistance by Upregulating Efflux Pump Hp1174 (gluP)', *Antimicrob Agents Chemother*, vol. 62, no. 11, Nov. 2018, doi: 10.1128/AAC.00957-18/SUPPL_FILE/ZAC011187608S1.PDF.

[355] R. Smiley, J. Bailey, M. Sethuraman, N. Posecion, and M. Showkat Ali, 'Comparative proteomics analysis of sarcosine insoluble outer membrane proteins from clarithromycin

resistant and sensitive strains of Helicobacter pylori', *Journal of Microbiology*, vol. 51, no. 5, pp. 612–618, Oct. 2013, doi: 10.1007/S12275-013-3029-5/METRICS.

[356] C. Hou, F. Yin, S. Wang, A. Zhao, Y. Li, and Y. Liu, 'Helicobacter pylori Biofilm-Related Drug Resistance and New Developments in Its Anti-Biofilm Agents', *Infect Drug Resist*, vol. 15, pp. 1561–1571, 2022, doi: 10.2147/IDR.S357473.

[357] K. Lewis, 'Multidrug tolerance of biofilms and persister cells', *Curr Top Microbiol Immunol*, vol. 322, pp. 107–131, 2008, doi: 10.1007/978-3-540-75418-3_6.

[358] D. E. Taylor, Z. Ge, D. Purych, T. Lo, and K. Hiratsuka, 'Cloning and sequence analysis of two copies of a 23S rRNA gene from Helicobacter pylori and association of clarithromycin resistance with 23S rRNA mutations', *Antimicrob Agents Chemother*, vol. 41, no. 12, pp. 2621–2628, 1997, doi: 10.1128/AAC.41.12.2621.

[359] A. T. Marques, J. M. B. Vítor, A. Santos, M. Oleastro, and F. F. Vale, 'Trends in Helicobacter pylori resistance to clarithromycin: from phenotypic to genomic approaches', *Microb Genom*, vol. 6, no. 3, p. e000344, 2020, doi: 10.1099/MGEN.0.000344.

[360] J. L. Pernodet, F. Boccard, M. T. Alegre, M. H. Blondelet-Rouault, and M. Guérineau, 'Resistance to macrolides,

lincosamides and streptogramin type B antibiotics due to a mutation in an rRNA operon of Streptomyces ambofaciens', *EMBO J*, vol. 7, no. 1, pp. 277–282, 1988, doi: 10.1002/J.1460-2075.1988.TB02810.X.

[361] S. A. Chisholm, J. Dave, and C. A. Ison, 'High-Level Azithromycin Resistance Occurs in Neisseria gonorrhoeae as a Result of a Single Point Mutation in the 23S rRNA Genes', *Antimicrob Agents Chemother*, vol. 54, no. 9, pp. 3812–3816, Sep. 2010, doi: 10.1128/AAC.00309-10.

[362] T. Hu, N. Chitnis, D. Monos, and A. Dinh, 'Next-generation sequencing technologies: An overview', *Hum Immunol*, vol. 82, no. 11, pp. 801–811, Nov. 2021, doi: 10.1016/J.HUMIMM.2021.02.012.

[363] A. Edwards *et al.*, 'In-field metagenome and 16S rRNA gene amplicon nanopore sequencing robustly characterize glacier microbiota', *bioRxiv*, p. 073965, Jan. 2019, doi: 10.1101/073965.

[364] S. Behjati and P. S. Tarpey, 'What is next generation sequencing?', *Arch Dis Child Educ Pract Ed*, vol. 98, no. 6, p. 236, Dec. 2013, doi: 10.1136/ARCHDISCHILD-2013-304340.

[365] J. Eid *et al.*, 'Real-time DNA sequencing from single polymerase molecules', *Science*, vol. 323, no. 5910, pp. 133–138, Jan. 2009, doi: 10.1126/SCIENCE.1162986.

[366] S. Goodwin, J. D. McPherson, and W. R. McCombie, ‘Coming of age: ten years of next-generation sequencing technologies’, *Nature Reviews Genetics 2016 17:6*, vol. 17, no. 6, pp. 333–351, May 2016, doi: 10.1038/nrg.2016.49.

[367] B. Segerman, ‘The Most Frequently Used Sequencing Technologies and Assembly Methods in Different Time Segments of the Bacterial Surveillance and RefSeq Genome Databases’, *Front Cell Infect Microbiol*, vol. 10, p. 527102, Oct. 2020, doi: 10.3389/FCIMB.2020.527102.

[368] T. Ribarska, P. M. Bjørnstad, A. Y. M. Sundaram, and G. D. Gilfillan, ‘Optimization of enzymatic fragmentation is crucial to maximize genome coverage: a comparison of library preparation methods for Illumina sequencing’, *BMC Genomics*, vol. 23, no. 1, pp. 1–11, Dec. 2022, doi: 10.1186/S12864-022-08316-Y/FIGURES/2.

[369] ‘Next-Generation Sequencing (NGS) | Explore the technology’. Accessed: Dec. 03, 2024. [Online]. Available: https://emea.illumina.com/science/technology/next-generation-sequencing.html

[370] D. Sims, I. Sudbery, N. E. Ilott, A. Heger, and C. P. Ponting, ‘Sequencing depth and coverage: key considerations in genomic analyses’, *Nature Reviews Genetics 2014 15:2*, vol. 15, no. 2, pp. 121–132, Jan. 2014, doi: 10.1038/nrg3642.

[371] F. J. Poelwijk, M. Socolich, and R. Ranganathan, ‘Learning the pattern of epistasis linking genotype and phenotype in a protein’, *Nature Communications 2019 10:1*, vol. 10, no. 1, pp. 1–11, Sep. 2019, doi: 10.1038/s41467-019-12130-8.

[372] ‘The variables for NGS experiments: coverage, read length, multiplexing’. Accessed: Dec. 03, 2024. [Online]. Available: https://irepertoire.com/ngs-considerations-coverage-read-length-multiplexing/

[373] M. Jain *et al.*, ‘Nanopore sequencing and assembly of a human genome with ultra-long reads’, *Nature Biotechnology 2018 36:4*, vol. 36, no. 4, pp. 338–345, Jan. 2018, doi: 10.1038/nbt.4060.

[374] G. A. Logsdon, M. R. Vollger, and E. E. Eichler, ‘Long-read human genome sequencing and its applications’, *Nature Reviews Genetics 2020 21:10*, vol. 21, no. 10, pp. 597–614, Jun. 2020, doi: 10.1038/s41576-020-0236-x.

[375] A. H. Beckett, K. F. Cook, and S. C. Robson, ‘A pandemic in the age of next-generation sequencing’, *Biochem (Lond)*, vol. 43, no. 6, pp. 10–15, Dec. 2021, doi: 10.1042/BIO_2021_187.

[376] A. Pomerantz *et al.*, ‘Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building’,

Gigascience, vol. 7, no. 4, pp. 1–14, Apr. 2018, doi: 10.1093/GIGASCIENCE/GIY033.

[377] A. M. Wenger *et al.*, 'Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome', *Nature Biotechnology 2019 37:10*, vol. 37, no. 10, pp. 1155–1162, Aug. 2019, doi: 10.1038/s41587-019-0217-9.

[378] E. Afgan *et al.*, 'The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update', *Nucleic Acids Res*, vol. 46, no. W1, pp. W537–W544, Jul. 2018, doi: 10.1093/NAR/GKY379.

[379] 'Geneious | Bioinformatics Software for Sequence Data Analysis'. Accessed: Dec. 01, 2024. [Online]. Available: https://www.geneious.com/

[380] A. Nekrutenko and J. Taylor, 'Next-generation sequencing data interpretation: enhancing reproducibility and accessibility', *Nature Reviews Genetics 2012 13:9*, vol. 13, no. 9, pp. 667–672, Aug. 2012, doi: 10.1038/nrg3305.

[381] L. Tedersoo, M. Albertsen, S. Anslan, and B. Callahan, 'Perspectives and Benefits of High-Throughput Long-Read Sequencing in Microbial Ecology', *Appl Environ Microbiol*, vol. 87, no. 17, pp. e00626-21, Aug. 2021, doi: 10.1128/AEM.00626-21.

[382] L. Fang and K. Wang, ‘Polishing high-quality genome assemblies’, *Nature Methods 2022 19:6*, vol. 19, no. 6, pp. 649–650, May 2022, doi: 10.1038/s41592-022-01515-1.

[383] J. Y. Lee *et al.*, ‘Comparative evaluation of Nanopore polishing tools for microbial genome assembly and polishing strategies for downstream analysis’, *Scientific Reports 2021 11:1*, vol. 11, no. 1, pp. 1–11, Oct. 2021, doi: 10.1038/s41598-021-00178-w.

[384] ‘GitHub - nanoporetech/medaka: Sequence correction provided by ONT Research’. Accessed: Nov. 24, 2024. [Online]. Available: https://github.com/nanoporetech/medaka

[385] R. Vaser, I. Sović, N. Nagarajan, and M. Šikić, ‘Fast and accurate de novo genome assembly from long uncorrected reads’, *Genome Res*, vol. 27, no. 5, pp. 737–746, May 2017, doi: 10.1101/GR.214270.116.

[386] C. Lee, C. Grasso, and M. F. Sharlow, ‘Multiple sequence alignment using partial order graphs’, *Bioinformatics*, vol. 18, no. 3, pp. 452–464, Mar. 2002, doi: 10.1093/BIOINFORMATICS/18.3.452.

[387] B. P. Alcock *et al.*, ‘CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database’, *Nucleic Acids*

Res, vol. 51, no. D1, pp. D690–D699, Jan. 2023, doi: 10.1093/NAR/GKAC920.

[388] N. Bonin *et al.*, 'MEGARes and AMR++, v3.0: an updated comprehensive database of antimicrobial resistance determinants and an improved software pipeline for classification using high-throughput sequencing', *Nucleic Acids Res*, vol. 51, no. D1, pp. D744–D752, Jan. 2023, doi: 10.1093/NAR/GKAC1047.

[389] A. F. Florensa, R. S. Kaas, P. T. L. C. Clausen, D. Aytan-Aktug, and F. M. Aarestrup, 'ResFinder – an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes', *Microb Genom*, vol. 8, no. 1, p. 000748, 2022, doi: 10.1099/MGEN.0.000748.

[390] B. Fernández-Caso, A. Miqueleiz, and T. Alarcón, 'Whole Genome Sequencing for Studying Helicobacter pylori Antimicrobial Resistance', *Antibiotics*, vol. 12, no. 7, p. 1135, Jun. 2023, doi: 10.3390/ANTIBIOTICS12071135.

[391] 'Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data'. Accessed: Nov. 24, 2024. [Online]. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[392] P. Ewels, M. Magnusson, S. Lundin, and M. Käller, ‘MultiQC: summarize analysis results for multiple tools and samples in a single report’, *Bioinformatics*, vol. 32, no. 19, pp. 3047–3048, Oct. 2016, doi: 10.1093/BIOINFORMATICS/BTW354.

[393] ‘GitHub - rrwick/Porechop: adapter trimmer for Oxford Nanopore reads’. Accessed: Nov. 25, 2024. [Online]. Available: https://github.com/rrwick/Porechop

[394] ‘GitHub - wdecoster/NanoPlot: Plotting scripts for long read sequencing data’. Accessed: Dec. 10, 2024. [Online]. Available: https://github.com/wdecoster/NanoPlot

[395] S. Chen, Y. Zhou, Y. Chen, and J. Gu, ‘fastp: an ultra-fast all-in-one FASTQ preprocessor’, *bioRxiv*, p. 274100, Apr. 2018, doi: 10.1101/274100.

[396] ‘bgruening/galaxytools: September release 2019’, doi: 10.5281/ZENODO.1419255.

[397] ‘GitHub - rrwick/Filtlong: quality filtering tool for long reads’. Accessed: Nov. 25, 2024. [Online]. Available: https://github.com/rrwick/Filtlong

[398] Y. Lin, J. Yuan, M. Kolmogorov, M. W. Shen, M. Chaisson, and P. A. Pevzner, ‘Assembly of long error-prone reads using de Bruijn graphs’, *Proc Natl Acad Sci U S A*, vol. 113, no. 52, pp. E8396–E8405, Dec. 2016, doi:

10.1073/PNAS.1604560113/SUPPL_FILE/PNAS.1604560113.SAPP.PDF.

[399] A. Mikheenko, A. Prjibelski, V. Saveliev, D. Antipov, and A. Gurevich, ‘Versatile genome assembly evaluation with QUAST-LG’, *Bioinformatics*, vol. 34, no. 13, pp. i142–i150, Jul. 2018, doi: 10.1093/BIOINFORMATICS/BTY266.

[400] ‘GitHub - galaxyproject/tools-iuc: Tool Shed repositories maintained by the Intergalactic Utilities Commission’. Accessed: Nov. 24, 2024. [Online]. Available: https://github.com/galaxyproject/tools-iuc

[401] R. R. Wick, M. B. Schultz, J. Zobel, and K. E. Holt, ‘Bandage: interactive visualization of de novo genome assemblies’, *Bioinformatics*, vol. 31, no. 20, pp. 3350–3352, Oct. 2015, doi: 10.1093/BIOINFORMATICS/BTV383.

[402] S. Hiltemann *et al.*, ‘Galaxy Training: A powerful framework for teaching!’, *PLoS Comput Biol*, vol. 19, no. 1, p. e1010752, Jan. 2023, doi: 10.1371/JOURNAL.PCBI.1010752.

[403] ‘Hands-on: Large genome assembly and polishing / Large genome assembly and polishing / Assembly’. Accessed: Nov. 25, 2024. [Online]. Available: https://training.galaxyproject.org/training-material/topics/assembly/tutorials/largegenome/tutorial.html

[404] T. Seemann, ‘Prokka: rapid prokaryotic genome annotation’, *Bioinformatics*, vol. 30, no. 14, pp. 2068–2069, Jul. 2014, doi: 10.1093/BIOINFORMATICS/BTU153.

[405] A. J. Page *et al.*, ‘Roary: rapid large-scale prokaryote pan genome analysis’, *Bioinformatics*, vol. 31, no. 22, pp. 3691–3693, Nov. 2015, doi: 10.1093/BIOINFORMATICS/BTV421.

[406] A. Stamatakis, ‘RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies’, *Bioinformatics*, vol. 30, no. 9, pp. 1312–1313, May 2014, doi: 10.1093/BIOINFORMATICS/BTU033.

[407] J. Hadfield, N. J. Croucher, R. J. Goater, K. Abudahab, D. M. Aanensen, and S. R. Harris, ‘Phandango: an interactive viewer for bacterial population genomics’, *Bioinformatics*, vol. 34, no. 2, pp. 292–293, Jan. 2018, doi: 10.1093/bioinformatics/btx610.

[408] Y. Fang *et al.*, ‘Whole-genome sequencing analyses and antibiotic resistance situation of 48 Helicobacter pylori strains isolated in Zhejiang, China’, *Gut Pathog*, vol. 16, no. 1, pp. 1–12, Dec. 2024, doi: 10.1186/S13099-024-00656-2/TABLES/6.

[409] B. Bogaerts *et al.*, ‘Closing the gap: Oxford Nanopore Technologies R10 sequencing allows comparable results to Illumina sequencing for SNP-based outbreak investigation of bacterial pathogens’, *J Clin Microbiol*, vol. 62, no. 5, May

2024, doi: 10.1128/JCM.01576-23/SUPPL_FILE/JCM.01576-23-S0001.DOCX.

[410] H. A. Safar, F. Alatar, and A. S. Mustafa, ‘Three Rounds of Read Correction Significantly Improve Eukaryotic Protein Detection in ONT Reads’, *Microorganisms*, vol. 12, no. 2, p. 247, Feb. 2024, doi: 10.3390/MICROORGANISMS12020247/S1.

[411] E. Berthenet *et al.*, ‘A GWAS on Helicobacter pylori strains points to genetic variants associated with gastric cancer risk’, *BMC Biol*, vol. 16, no. 1, pp. 1–11, Aug. 2018, doi: 10.1186/S12915-018-0550-3/FIGURES/4.

[412] D. Antipov, A. Korobeynikov, J. S. McLean, and P. A. Pevzner, ‘hybridSPAdes: an algorithm for hybrid assembly of short and long reads’, *Bioinformatics*, vol. 32, no. 7, pp. 1009–1015, Apr. 2016, doi: 10.1093/BIOINFORMATICS/BTV688.

[413] E. L. van Dijk, Y. Jaszczyszyn, D. Naquin, and C. Thermes, ‘The Third Revolution in Sequencing Technology’, *Trends in Genetics*, vol. 34, no. 9, pp. 666–681, Sep. 2018, doi: 10.1016/J.TIG.2018.05.008.

[414] K. Howe *et al.*, ‘Significantly improving the quality of genome assemblies through curation’, *Gigascience*, vol. 10, no. 1, pp. 1–9, Jan. 2021, doi: 10.1093/GIGASCIENCE/GIAA153.

[415] R. R. Wick, L. M. Judd, C. L. Gorrie, and K. E. Holt, 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLoS Comput Biol*, vol. 13, no. 6, Jun. 2017, doi: 10.1371/JOURNAL.PCBI.1005595.

[416] D. Falush, 'The Remarkable Genetics of Helicobacter pylori', *mBio*, vol. 13, no. 6, pp. e02158-22, Dec. 2022, doi: 10.1128/MBIO.02158-22.

[417] P. Cuber *et al.*, 'Comparing the accuracy and efficiency of third generation sequencing technologies, Oxford Nanopore Technologies, and Pacific Biosciences, for DNA barcode sequencing applications', *Ecol Genet Genom*, vol. 28, p. 100181, Sep. 2023, doi: 10.1016/J.EGG.2023.100181.

[418] P. J. Jenks and D. I. Edwards, 'Metronidazole resistance in Helicobacter pylori', *Int J Antimicrob Agents*, vol. 19, no. 1, pp. 1–7, Jan. 2002, doi: 10.1016/S0924-8579(01)00468-X.

[419] A. Goodwin, D. Kersulyte, G. Sisson, S. J. O. Veldhuyzen Van Zanten, D. E. Berg, and P. S. Hoffman, 'Metronidazole resistance in Helicobacter pylori is due to null mutations in a gene (rdxA) that encodes an oxygen-insensitive NADPH nitroreductase', *Mol Microbiol*, vol. 28, no. 2, pp. 383–393, Apr. 1998, doi: 10.1046/J.1365-2958.1998.00806.X.

[420] E. Kouhsari *et al.*, 'Heteroresistance to clarithromycin and metronidazole in patients with a Helicobacter pylori infection:

a systematic review and meta-analysis', *Ann Clin Microbiol Antimicrob*, vol. 21, no. 1, pp. 1–8, Dec. 2022, doi: 10.1186/S12941-022-00509-3/FIGURES/3.

[421] M. Ismail *et al.*, 'Molecular detection of Helicobacter pylori and its genotypic antimicrobial resistance patterns in dyspeptic Mozambican patients', *Helicobacter*, vol. 28, no. 4, p. e13000, Aug. 2023, doi: 10.1111/HEL.13000.

[422] E. J. Gong *et al.*, 'Genotypic and Phenotypic Resistance to Clarithromycin in Helicobacter pylori Strains', *Journal of Clinical Medicine 2020, Vol. 9, Page 1930*, vol. 9, no. 6, p. 1930, Jun. 2020, doi: 10.3390/JCM9061930.

[423] D. Azzaya *et al.*, 'High Antibiotic Resistance of Helicobacter pylori and Its Associated Novel Gene Mutations among the Mongolian Population', *Microorganisms 2020, Vol. 8, Page 1062*, vol. 8, no. 7, p. 1062, Jul. 2020, doi: 10.3390/MICROORGANISMS8071062.

[424] R. Caliskan *et al.*, 'Antimicrobial resistance of *Helicobacter pylori* strains to five antibiotics, including levofloxacin, in Northwestern Turkey', *Rev Soc Bras Med Trop*, vol. 48, no. 3, pp. 278–284, Jun. 2015, doi: 10.1590/0037-8682-0027-2015.

[425] A. J. Matta, D. C. Zambrano, and A. J. Pazos, 'Punctual mutations in 23S rRNA gene of clarithromycin-resistant

Helicobacter pylori in Colombian populations', *World J Gastroenterol*, vol. 24, no. 14, p. 1531, Apr. 2018, doi: 10.3748/WJG.V24.I14.1531.

[426] R. A. Hussein, M. T. S. Al-Ouqaili, and Y. H. Majeed, 'Detection of clarithromycin resistance and 23SrRNA point mutations in clinical isolates of Helicobacter pylori isolates: Phenotypic and molecular methods', *Saudi J Biol Sci*, vol. 29, no. 1, p. 513, Jan. 2021, doi: 10.1016/J.SJBS.2021.09.024.

[427] F. Mégraud and P. Lehours, 'Helicobacter pylori Detection and Antimicrobial Susceptibility Testing', *Clin Microbiol Rev*, vol. 20, no. 2, p. 280, Apr. 2007, doi: 10.1128/CMR.00033-06.

[428] R. I. Alfaray *et al.*, 'Global Antimicrobial Resistance Gene Study of Helicobacter pylori: Comparison of Detection Tools, ARG and Efflux Pump Gene Analysis, Worldwide Epidemiological Distribution, and Information Related to the Antimicrobial-Resistant Phenotype', *Antibiotics*, vol. 12, no. 7, p. 1118, Jul. 2023, doi: 10.3390/ANTIBIOTICS12071118/S1.

[429] C. A. Arias, J. Weisner, J. M. Blackburn, and P. E. Reynolds, 'Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in Enterococcus gallinarum BM4174', *Microbiology (Reading)*, vol. 146 (Pt 7), no. 7, pp. 1727–1734, 2000, doi: 10.1099/00221287-146-7-1727.

[430] S. Donadio and M. Sosio, ‘Glycopeptides, Antimicrobial’, *Encyclopedia of Microbiology, Third Edition*, pp. 455–471, Jan. 2009, doi: 10.1016/B978-012373944-5.00040-7.

[431] C. yu Chen *et al.*, ‘Detection of Antimicrobial Resistance Using Proteomics and the Comprehensive Antibiotic Resistance Database: A Case Study’, *Proteomics Clin Appl*, vol. 14, no. 4, p. 1800182, Jul. 2020, doi: 10.1002/PRCA.201800182.

[432] ‘National Database of Antibiotic Resistant Organisms (NDARO) - Pathogen Detection - NCBI’. Accessed: Dec. 30, 2024. [Online]. Available: https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/

[433] E. Zankari, R. Allesøe, K. G. Joensen, L. M. Cavaco, O. Lund, and F. M. Aarestrup, ‘PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens’, *Journal of Antimicrobial Chemotherapy*, vol. 72, no. 10, pp. 2764–2768, Oct. 2017, doi: 10.1093/JAC/DKX217.

[434] M. Papp and N. Solymosi, ‘Review and Comparison of Antimicrobial Resistance Gene Databases’, *Antibiotics*, vol. 11, no. 3, p. 339, Mar. 2022, doi: 10.3390/ANTIBIOTICS11030339/S1.

[435] S. K. Gupta *et al.*, 'ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes', *Antimicrob Agents Chemother*, vol. 58, no. 1, pp. 212–220, Jan. 2014, doi: 10.1128/AAC.01310-13.

[436] E. Doster *et al.*, 'MEGARes 2.0: a database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data', *Nucleic Acids Res*, vol. 48, no. D1, pp. D561–D569, Jan. 2020, doi: 10.1093/NAR/GKZ1010.

[437] V. Bortolaia *et al.*, 'ResFinder 4.0 for predictions of phenotypes from genotypes', *Journal of Antimicrobial Chemotherapy*, vol. 75, no. 12, pp. 3491–3500, Dec. 2020, doi: 10.1093/JAC/DKAA345.

[438] A. Edalatmand and A. G. McArthur, 'CARD*Shark: automated prioritization of literature curation for the Comprehensive Antibiotic Resistance Database', *Database*, vol. 2023, Aug. 2023, doi: 10.1093/DATABASE/BAAD023.

[439] R. A. Jensen, 'Orthologs and paralogs - we need to get it right', *Genome Biology 2001 2:8*, vol. 2, no. 8, pp. 1–3, Aug. 2001, doi: 10.1186/GB-2001-2-8-INTERACTIONS1002.

[440] H. Su *et al.*, 'Evolutionary mechanism leading to the multi-cagA genotype in Helicobacter pylori', *Scientific Reports 2019*

9:1, vol. 9, no. 1, pp. 1–13, Aug. 2019, doi: 10.1038/s41598-019-47240-2.

[441] T. Mehrotra *et al.*, 'Antimicrobial resistance and virulence in Helicobacter pylori: Genomic insights', *Genomics*, vol. 113, no. 6, pp. 3951–3966, Nov. 2021, doi: 10.1016/J.YGENO.2021.10.002.

[442] D. H. S. Block, R. Hussein, L. W. Liang, and H. N. Lim, 'Regulatory consequences of gene translocation in bacteria', *Nucleic Acids Res*, vol. 40, no. 18, p. 8979, Oct. 2012, doi: 10.1093/NAR/GKS694.

[443] M. Nambiar and S. C. Raghavan, 'How does DNA break during chromosomal translocations?', *Nucleic Acids Res*, vol. 39, no. 14, p. 5813, Aug. 2011, doi: 10.1093/NAR/GKR223.

[444] K. Yahara, P. Lehours, and F. F. Vale, 'Analysis of genetic recombination and the pan-genome of a highly recombinogenic bacteriophage species', *Microb Genom*, vol. 5, no. 8, p. e000282, Aug. 2019, doi: 10.1099/MGEN.0.000282/CITE/REFWORKS.

[445] Y. Furuta, K. Yahara, M. Hatakeyama, and I. Kobayashi, 'Evolution of cagA Oncogene of Helicobacter pylori through Recombination', *PLoS One*, vol. 6, no. 8, p. e23499, 2011, doi: 10.1371/JOURNAL.PONE.0023499.

[446] A. Kim *et al.*, ‘Helicobacter pylori bab Paralog Distribution and Association with cagA, vacA, and homA/B Genotypes in American and South Korean Clinical Isolates’, *PLoS One*, vol. 10, no. 8, p. e0137078, Aug. 2015, doi: 10.1371/JOURNAL.PONE.0137078.

[447] ‘Bacterial Antimicrobial Resistance Reference Gene ... (ID 313047) - BioProject - NCBI’. Accessed: Dec. 30, 2024. [Online]. Available: https://www.ncbi.nlm.nih.gov/bioproject/313047

[448] A. M. Varani, P. Siguier, E. Gourbeyre, V. Charneau, and M. Chandler, ‘ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes’, *Genome Biol*, vol. 12, no. 3, pp. 1–9, Mar. 2011, doi: 10.1186/GB-2011-12-3-R30/FIGURES/4.

[449] C. Yang, Y. Cui, X. Didelot, R. Yang, and D. Falush, ‘Why panmictic bacteria are rare’, *bioRxiv*, p. 385336, Jan. 2019, doi: 10.1101/385336.

[450] A. Sukri *et al.*, ‘Multidrug-Resistant Helicobacter pylori Strains: A Five-Year Surveillance Study and Its Genome Characteristics’, *Antibiotics*, vol. 11, no. 10, p. 1391, Oct. 2022, doi: 10.3390/ANTIBIOTICS11101391/S1.

[451] A. Alvarez-Aldana *et al.*, ‘Genomic insights into the antimicrobial resistance and virulence of Helicobacter pylori isolates from gastritis patients in Pereira, Colombia’, *BMC Genomics*, vol. 25, no. 1, pp. 1–12, Dec. 2024, doi: 10.1186/S12864-024-10749-6/FIGURES/2.

[452] F. Alleweldt *et al.*, ‘Economic evaluation of whole genome sequencing for pathogen identification and surveillance - results of case studies in Europe and the Americas 2016 to 2019’, *Eurosurveillance*, vol. 26, no. 9, p. 1900606, Mar. 2021, doi: 10.2807/1560-7917.ES.2021.26.9.1900606/CITE/REFWORKS.

[453] E. Tshibangu-Kabamba and Y. Yamaoka, ‘Helicobacter pylori infection and antibiotic resistance — from biology to clinical implications’, *Nature Reviews Gastroenterology & Hepatology 2021 18:9*, vol. 18, no. 9, pp. 613–629, May 2021, doi: 10.1038/s41575-021-00449-x.

[454] R. Allison, D. M. Lecky, M. Bull, K. Turner, G. Godbole, and C. A. M. McNulty, ‘Audit of Helicobacter pylori Testing in Microbiology Laboratories in England: To Inform Compliance with NICE Guidance and the Feasibility of Routine Antimicrobial Resistance Surveillance’, *Int J Microbiol*, vol. 2016, no. 1, p. 8540904, Jan. 2016, doi: 10.1155/2016/8540904.

[455] N. Noguchi *et al.*, ‘Detection of mixed clarithromycin-resistant and -susceptible Helicobacter pylori using nested PCR and direct sequencing of DNA extracted from faeces’, *J Med Microbiol*, vol. 56, no. 9, pp. 1174–1180, Sep. 2007, doi: 10.1099/JMM.0.47302-0/CITE/REFWORKS.

[456] E. Cambau *et al.*, ‘Evaluation of a new test, GenoType HelicoDR, for molecular detection of antibiotic resistance in Helicobacter pylori’, *J Clin Microbiol*, vol. 47, no. 11, pp. 3600–3607, 2009, doi: 10.1128/JCM.00744-09/ASSET/C51A9D6C-9322-48A5-8FD4-8F13782D6239/ASSETS/GRAPHIC/ZJM0110992690002.JPEG.

[457] K. R. Kumar, M. J. Cowley, and R. L. Davis, ‘Next-Generation Sequencing and Emerging Technologies’, *Semin Thromb Hemost*, vol. 50, no. 07, pp. 1026–1038, May 2024, doi: 10.1055/S-0044-1786397/ID/JR501112-15/BIB.

[458] J. Sunuwar and R. K. Azad, ‘A machine learning framework to predict antibiotic resistance traits and yet unknown genes underlying resistance to specific antibiotics in bacterial strains’, *Brief Bioinform*, vol. 22, no. 6, pp. 1–8, Nov. 2021, doi: 10.1093/BIB/BBAB179.

[459] M. AlQuraishi, ‘Machine learning in protein structure prediction’, *Curr Opin Chem Biol*, vol. 65, pp. 1–8, Dec. 2021, doi: 10.1016/J.CBPA.2021.04.005.

[460] S. Bertagnolio *et al.*, ‘WHO global research priorities for antimicrobial resistance in human health’, *Lancet Microbe*, vol. 5, no. 11, Nov. 2024, doi: 10.1016/S2666-5247(24)00134-4.