The use of lytic plasmid-associated sex pilus-specific bacteriophages to drive microbial evolution towards loss of virulence

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Visara Chit-Opas
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Supervised by Associated Professor Michael Jones

School of Veterinary Medicine and Science
University of Nottingham

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Abstract

Due to an increasing of antibiotic resistance prevalence, several alternatives to antibiotics have been considered, including bacteriophages. This project investigates the potential of sex pilus-specific phage MS2 to drive loss and horizontal transfer of virulence plasmids from *E. coli*. The study assessed new *E. coli* host range of phage MS2, determining the natural transfer rate of pHaemolysin, pColicin V and F plasmids, and investigating the impact of phage infection on the virulence plasmids. Phage MS2 could not infect or replicate on the *E. coli* strains assessed in this study. The horizontal transfer rate of the pHaemolysin and pColicin V plasmids was negligible. Expression of the F pili would be essential for phage susceptibility and bacterial conjugation. Therefore, it is essential to investigate level of the F pili expression within the virulence plasmids. In conclusion, there is a potential limitation in using F pilus-specific bacteriophage to drive loss of virulence plasmids and this we propose this is due to low level of F pili expression and phage susceptibility.

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Abbreviation list

Amp	Ampicillin
cAMP	cyclic Adenosine Monophosphate
AMR	Antimicrobial Resistance
APEC	Avian Pathogenic *E. coli*
CF	Colonisation Factor
cGMP	cyclic Guanosine Monophosphate
ColV	Colicin V
DAEC	Diffusely Adherent *E. coli*
DNA	Deoxyribonucleic Acid
DW	distilled water
EAEC	Enteroaggregative *E. coli*
EAF	Enteropathogenic *E. coli* Adherence factor
EDTA	Ethylenediaminetetraacetic Acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extra-intestinal Pathogenic *E. coli*
F plasmid, pilus (pili)	Fertility plasmid, pilus (pili)
GC-content	Guanine-cytosine content
Hly	Haemolysin
HC	Haemorrhagic colitis
HUS	Haemolytic-Uremic Syndrome
IS	Insertion Sequence
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharide
LT	Heat-labile Enterotoxin
MDR	Multidrug-resistant
MNEC	Meningitis-associated *E. coli*

MOB Plasmid Mobility

MOI Multiplicity of Infection

MPF Mating Pair Formation

NTEC Necrotoxigenic *E. coli*

ORF Open Reading Frame

PAI Pathogenicity Island

PBS Phosphate-buffered Saline

PCR Polymerase Chain Reaction

PFU Plaque Forming Unit

PG Peptidoglycan

R plasmid, factor Resistance plasmid, factor

Rif Rifampicin

RNA Ribonucleic Acid

RPBs Receptor Binding Proteins

RTX Repeats in Toxin

SBA Sheep Blood Agar

SD Standard Deviation

ST Heat-stable Enterotoxin

STEC Shiga Toxin-producing *E. coli*

UPEC Uropathogenic *E. coli*

UTI Urinary Tract Infection

VT Verotoxin

VTEC Verotoxin-producing *E. coli*

1 Introduction

1.1 *Escherichia coli*

E. coli strains are commensal residents which reside in mucous layer of the mammalian colon and only cause disease in immunocompromised hosts or the hosts that gastrointestinal barriers are broken. Some strains have acquired specific virulence features, conferring increased ability to adapt to new niches and allowing them to cause a broad spectrum of disease (Kaper, Nataro, and Mobley 2004). Most of the virulence traits are encoded on mobile genetic elements that can be transferred between different strains or recombine into the genome, creating new combinations of virulence factors. The most successful combinations of virulence factors have persisted and led to specific pathotypes of *E. coli*, capable of causing disease in susceptible hosts as following; enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis.

Virulent strains of *E. coli* can be categorised into those that cause intestinal or extra-intestinal infections (ExPEC) (Nataro and Kaper 1998). These pathogenic *E. coli* strains can be further classified into pathotypes based on their pathology and the presence of specific virulence plasmids (Table 1.1) (Kaper, Nataro, and Mobley 2004). There are 6 intestinal pathogenic strains; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli (*DAEC). Whereas, ExPEC strains consist of uropathogenic *E. coli* (UPEC), meningitis-associated *E. coli* (MNEC) and avian pathogenic *E. coli* (APEC) which causes extra-intestinal infections – urinary tract and respiratory infections, pericarditis and septicaemia (Kaper, Nataro, and Mobley 2004; Nataro and Kaper 1998).

EPEC, EHEC and ETEC can also cause disease in animals in a similar manner to human infections. With the exception of the intracellular pathogen EIEC pathogenic *E. coli* replicate extracellularly (Kaper, Nataro, and Mobley 2004). The *E. coli* pathotypes are further classified by their surface antigens, O (lipopolysaccharide, LPS) and H (flagellar), into serogroups (O antigen only) or serotypes (O and H antigens) (Neidhardt and Curtiss 1996; Nataro and Kaper 1998).

Table 1.1 Association of pathotype, disease and virulence-associated plasmid(s) modified from Johnson and Nolan 2009 and Kaper, Nataro, and Mobley 2004

E. coli pathotype	Anatomical pathotype	Disease	Virulence-associated plasmid(s)
Enteropathogenic (EPEC)	intestinal	Non-bloody diarrhoea	Coli adherence factor (EAF) plasmid
Enterotoxigenic (ETEC)	intestinal	Watery diarrhoea	Plasmid encoding colonization factors and toxins
Enteroaggregative (EAEC)	intestinal	Persistent diarrhoea	Plasmid encoding adherence factors and toxins
Enteroinvasive (EIEC)	intestinal	Watery diarrhoea, inflammatory colitis, or dysentery	Invasion plasmid
Enterohaemorrhagic (EHEC)	intestinal	Non-bloody diarrhoea, haemorrhagic colitis (HC), or haemolytic-uremic syndrome (HUS)	Plasmid encoding toxins (pO157)
Diffusely adherent (DAEC)	intestinal	Non-bloody diarrhoea	Plasmids encoding adhesins
Extraintestinal pathogenic (ExPEC)[a]	extraintestinal	UTI, sepsis, neonatal meningitis, or avian colibacillosis	ColV and Vir plasmids
Necrotoxigenic (NTEC)	intestinal/extraintestinal	UTI, septicaemia, diarrhoea	Vir plasmid

[a]ExPEC includes uropathogenic (UPEC), avian pathogenic (APEC) and meningitis-associated *E. coli* (MNEC).

There is an increasing problem with antimicrobial resistance (AMR) *E. coli* in hospital settings. During 2012 to 2013, high percentages of multidrug-resistant (MDR) *E. coli* infections and death rates from bacteria resistance were reported (World Health Organization 2017). Underestimating the impact of AMR can cause a huge economic loss and contamination of AMR *E. coli* in livestock products can lead to *E. coli*-associated diseases in human (Russo and Johnson 2003; Lee et al. 2009). This increase in AMR has led to the need for alternatives none antibiotic therapies or strategies to reduce AMR in bacteria. The alternatives such as probiotics, antimicrobial peptides, lysins and bacteriophages have been used not only to mitigate the AMR issue but also to target virulence factors of the bacteria which mostly encoded by plasmids (Ghosh et al. 2019). Using sex pilus-specific bacteriophages of *E. coli* to interfere with their plasmid-encoded virulence factors is

a promising method of interest to prevent pathogenic *E. coli* to transfer their pathogenic traits. Understanding plasmid biology and plasmids encoding virulence factors as well as sex pilus-specific phages biology could be beneficial in investigating effects of the phages on *E. coli* virulence factors which are discussed below.

1.2 Plasmid biology

1.2.1 Plasmid classification

Plasmids are self-replicating dsDNA episomal elements with their own origins of replication, being physically separated from the main chromosome (Clowes 1972; Watson 1987). They usually encode non-essential functions that are not affect the growth of bacteria so that neither their existence nor absence affect survival of the bacteria. Many plasmids are expendable; however, they provide unique functions to bacteria. Their copy number can differ from one plasmid to up to fifty per bacterium. This difference in copy number can be reflected in expression levels of their protein products (Watson 1987).

Plasmids can be classified based on several criteria such as host-tropism, features of replication and conjugation (mating pair formation; MPF), and plasmid mobility (MOB classification system) (Garcillan-Barcia, Alvarado, and de la Cruz 2011; Shintani, Sanchez, and Kimbara 2015; Smillie et al. 2010). There are two types of transmissible plasmids; conjugative and mobilizable plasmids. The conjugative plasmid contains the specific secretion system and coupling protein, conferring conjugation process. While, the mobilizable plasmid have an incomplete transfer system lacking the secretion system and sometimes the coupling protein and require co-expression of these genes from a conjugative plasmid to facilitate in plasmid transfer (Drlica and Gennaro 2001; Alvarado, Garcillan-Barcia, and de la Cruz 2012).

Plasmid incompatibility classification is another method used to classify plasmids into incompatibility groups based on their replication control (Novick and Hoppensteadt 1978; Novick 1987; Ishii, Hashimoto-Gotoh, and Matsubara 1978). Currently, there are 22 incompatibility groups of plasmids among the Enterobacteriaceae (Table 1.2) (Couturier et al. 1988; Carattoli et al. 2005; Carattoli 2009). Plasmids with the same replication control are incompatible, while plasmids with different replication controls are compatible (Couturier et al. 1988). Incompatible

plasmids cannot reside and maintain within the same bacterium or both be stably inherited in the absence of external selection because they share elements of their replication and partitioning systems. As a consequence, one of the two plasmids will be lost due to fluctuations in their copy numbers (Novick et al. 1976; Novick 1987). Incompatible plasmids can also exhibit entry exclusion which is a change on the surface of plasmid-containing cells in order to inhibit the transport of a related plasmid (Couturier et al. 1988).

Incompatibility groups can be identified through PCR-based inc/rep typing method by using multiplex- and simplex-PCRs primer pairs to detect specific sites of each replicon such as iterons which encode replication initiation proteins, RNAI and *repA* genes (Carattoli et al. 2005; Konieczny et al. 2014). There are possibilities that one plasmid can carry multiple replicons and replicons can include various different loci which are not universal across plasmids. Therefore, MOB typing system is also applied to detect relaxase protein of the plasmid which is responsible for mobility and presented solely per plasmid (del Solar et al. 1998; Orlek et al. 2017).

Table 1.2 Incompatibility groups and examples of plasmid and their species of origin (Carattoli 2009; Carattoli et al. 2005; Couturier et al. 1988)

Incompatibility (Inc) groups	Plasmid examples	Species of origin	Plasmid function
FIA	F' lac	*E. coli*	Fertility
FIB	F' lac, ColV	*E. coli*	Fertility, Colicin V production
FIC	F' lac, ColV	*E. coli*	Fertility, Colicin V production
HI1	R27	*S.* Typhi	Drug resistance Tc
HI2	R478	*S. marcescens*	Drug resistance CmKmTcHgTe
I1	R483	*E. coli*	Drug resistance SmSpTp
Iγ	R621a	*S.* Typhimurium	Drug resistance Tc
L/M	pSem	*S.* Typhimurium	Drug resistance GmAkKmSu and expanded-spectrum cephalosporins (Villa et al. 2000)
N	N3	*Shigella* spp.	Drug resistance SmSpSuTcHg

P	RP4	*P. aeruginosa*	Drug resistance ApKmTc
W	pSa	*S. flexneri*	Drug resistance CmGmKmSmSpSuTm
T	Rts1	*P. vulgaris*	Drug resistance Km
A/C	pIP40a	*P. aeruginosa*	Drug resistance ApKmSuHg
K	R387	*S. flexneri*	Drug resistance CmSm
B/O	R16	*E. coli*	Drug resistance ApSmSpSuTc
X	R6K		Drug resistance ApSm
Y	MIP231		Drug resistance Tc
FIIA	R1*drd*-19, ColV	*E. coli* (Molin et al. 1979)	Drug resistance ApCmKmSmSpSu
FII	O157	*E. coli* O157:H7	Virulence factors production (Lim et al. 2007)
FIII	ColB-K98	*E. coli*	Colicin B and M production (Cooper and Rowbury 1986)
FIV and variants	R124	*S.* Typhimurium	Drug resistance

Abbreviations for drug resistance markers: Ak, amikacin; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline; Sm, streptomycin; Su, sulfonamides; Sp, spectinomycin; Gm, gentamycin; Hg, mercuric ions; Te, tellurium; Tp, trimethoprim; Tm, tobramycin

1.2.2 Plasmid transfer mechanisms

Plasmids can be transfer horizontally through main mechanisms; transformation and conjugation (Smillie et al. 2010). These mechanisms confer widespread transfer of genes concerning antibiotic resistance, metabolic functions and virulence determinants (Babic et al. 2008). Moreover, plasmids can integrate partially or completely of the bacterial genome into themselves by genetic recombination, increasing probability of genetic transfer (Brinton 1971).

Transformation in the environment is a process where naturally competent bacteria can take up and incorporate linear, naked DNA. This then has the potential to recombine into their own chromosome (Watson 1987).

Conjugation is a directed process of unidirectional transfer mediated by F plasmid from a F^+ plasmids containing donor strain to recipient cell which do not carry F plasmid (F^- cell) without wall-to-wall contact (Babic et al. 2008; Ou and Anderson 1970). The F^- recipient cell converts into F^+ donor cell by receiving a copy of F plasmid from its donor (Griffiths et al. 1999). Transfer of F

plasmid is mediated by the FinOP fertility inhibition system which the antisense RNA (FinP) and the RNA-binding protein (FinO) control the expression of TraJ, the transcription factor that upregulates the *tra* operon containing genes required for conjugation and plasmid transfer. FinO binds to FinP to protect the FinP from degradation and promote duplex formation of FinP and *traJ* mRNA, resulting in repression of *traJ* expression and conjugative F transfer (Gubbins et al. 2003). However, the F plasmid is normally de-repressed due to IS3 insertion within *finO*, causing mutation of the finO which gives rise to expression of *tra* operon (Cheah and Skurray 1986; Frost and Koraimann 2010). This confers high frequency transfer (HFT) phenotype for transconjugants to disseminate cargo genes such as antibiotic resistance and virulence determinants at high frequency. Whereas, other F-like plasmids in natural environment have a repressed phenotype with reduced mating efficiency (Frost and Koraimann 2010). The de-repressed resistance (R) factor, a F-like plasmid, can confer ability to conjugate on F^- bacteria through F pilus production. Due to ability to inhibit F pili of the R factor, introduction of R factor into F^+ cell can considerably reduce conjugation of the F^+ strain by producing cytoplasmic repressor functioning on F pili, leading to loss of susceptibility to F-specific serum and F-specific phages (Ottow 1975). Genes within the F plasmid are responsible for mediating the contact between donor and recipient cells and regulation of DNA mobilization and unidirectional transfer. DNA transfer involves rolling circle replication, beginning with nicking of one strand of the F plasmid at *oriT* by the TraI protein, then the TraI becomes covalently bound to the 5' end of the plasmid DNA. The plasmid is unwound by TraI and the nicked strand is transferred to the recipient cell (Drlica and Gennaro 2001). After the process is completed, transferred single-stranded DNA converted to double-stranded DNA by the synthesis of the complementary strand in the recipient cell in F' transfer or incorporated into the recipient chromosome or degraded by RecBCD exonuclease (helicase/nuclease; enzyme degrading single- and double-stranded linear DNA) in Hfr transfer (Eggleston and West 1997; Kowalczykowski et al. 1994; Lloyd and Buckman 1995; Neidhardt and Curtiss 1996). The Hfr strain is attributed to the integration of F plasmid into the chromosome of the host cell after excision of F into F' molecule carrying chromosomal genes with the conjugation genes which confer the chromosome genes to be mobilized and transferred to a recipient (Low 1991). Therefore, both Hfr and F' transfers can serve as DNA vehicles in horizontal gene transfer between bacteria (Babic et al. 2008).

F plasmids contain 36 *tra* genes which encode the proteins required for conjugation, transfer and surface exclusion (Drlica and Gennaro 2001; Moore et al. 1987). These include ORFs for F pili (sex pili) which have an important role in single-stranded DNA transfer through conjugation by establishing contact between mating cells (Babic et al. 2008). The tips of the pili interact with the surface of cells lacking the F plasmid and then the F^- cells are drawn to the F^+ cells by depolymerisation (retraction) of the pili (Drlica and Gennaro 2001). A few theories of how DNA is transported through the F pili were proposed. First, DNA is believed to transported through the groove between the two rods of pili. Second, the F pili move resembling a conveyor belt while carrying DNA toward recipient. The last theory is that DNA bound to the F pilus, and then the F pilus moves toward recipient (Brinton 1971). Within the donor cell, the pili are double-stranded circular sex factor which can either replicate within the cell or contact with the recipient cell. Theirs active form is single strand for active transport to the recipient (Brinton 1971). Sex pili function to recognise recipient cell surfaces and conduct DNA transfer through cell-to-cell contact unidirectionally from the donor to its recipient. Normally, one donor cell can have 2-3 pili so it can attach to more than one recipient. Interaction between donors and recipients can be blocked through interaction of donor cell and F pilus-specific phage (Brinton 1971).

1.2.3 Conjugative pili (F pili)

Pili can be classified into 5 types based on functions and characteristics. The most common types are type I (common pili) and type II pili (conjugative or sex pili) (Brinton 1965; Ottow 1975). Sex pili originate from the donor membrane, they are assembled from F pilin molecule; phosphor-glycoprotein, which controlled by *traA*, *traQ* and *traX* genes on the F plasmid which are responsible for F pilin formation and maturation (Ohtsubo 1968; Brinton, Gemski, and Carnahan 1964; Frost, Ippen-Ihler, and Skurray 1994). *TraA* encodes the pilin, the pilus subunit of approximately 70 amino acids, while *traQ* encodes inner membrane protein which is necessary for pilin maturation. *TraX*, a highly conserved gene among F-like plasmids, encodes integral inner membrane protein with transmembrane domains responsible for acetylation at the N-terminus of F pilin (Frost, Ippen-Ihler, and Skurray 1994; Moore, Sowa, and Ippen-Ihler 1982; Maneewannakul, Maneewannakul, and Ippen-Ihler 1995). F pili grow rapidly and vertically from the cell surface up to one to two millimetres (Drlica and Gennaro 2001; Ottow 1975). The pili can detach from the cell by shearing which removes the ability to conjugate. However, F pili can return

to the normal number (ca. one per cell) and length within five minutes (Novotny et al. 1969; Novotny, Carnahan, and Brinton 1969; Brinton 1971). There are two forms of F pili; fibre and vesicle forms. Fibre form F pili assemble into rods that RNA pilus phage are adsorbed. Vesicle form F pili develop terminal knobs at one end of the F pilus, opposite to the site of RNA and DNA F pilus phage adsorption. The latest form rarely occurs but can be observed in F pili that had separated from donor cells and later re-adsorbed to donor cells by the distal ends with the vesicle on the proximal ends (Brinton 1971).

F pili cannot be removed with the treatments that damage the cell membrane such as lysozyme or EDTA; therefore, donors retain their ability (Brinton 1971). However, The F^+ cells are more susceptible to antibacterial agents such as sodium dodecyl sulphate, chloroform, lysozyme and tris-EDTA. This may be due to instability of the cell membrane in the presence of F pilin. Moreover, surface exclusion may occur when F pilin synthesis of one donor cell is interfered by another donor cell which finally leads to disappearance of F pili from the cell surface and loss of donor ability and pilus-specific phage sensitivity. Consequently, donors would become recipients (Brinton 1971). Besides, the presence of F pili also impairs flagellar function which requires membrane integrity. This gives rise to loss of motility of the F^+ cells (Skaar, Richter, and Lederberg 1957).

1.3 Virulence plasmids of *E. coli*

IncF plasmids were the first identified in enteric bacteria including *E. coli*, followed by the discovery of the colicinogenic (Col), antibiotic resistance (R) and virulence plasmids (Watson 1987; Blum et al. 1995; Gyles 2004). Virulence plasmids of *E. coli* for the most part are self-transmissible (Smillie et al. 2010; Gyles 2004) and the majority of virulence and antibiotic resistance (AMR) plasmids of *E. coli* are within the incompatibility group F (IncF) (Johnson and Nolan 2009; Kaper, Nataro, and Mobley 2004). However a number of virulence genes are found on incompatibility groups IncB/O (Kaper, Nataro, and Mobley 2004).

This study focuses on virulence plasmids within the incompatibility group F, including colicin V, haemolysin, sex pili, capsular pilus antigen, and enterotoxin. However, only the colicin V and

haemolysin activities were observed in the experiment because they can be easily observed on agar plate.

1.3.1 Colicin V

Colicin V (ColV) was first described as a heat-labile product from *E. coli* V (Gratia 1925). ColV is a peptide antibiotic affecting the cytoplasmic membrane of closely related species susceptible cells but not on the bacteriocin-producing strain (Jacob et al. 1953; Yang and Konisky 1984). The ColV is encoded on the colicinogenic plasmid (pCol), belongs to incompatibility groups FIB, FIC and FIIA, of colicinogenic strains of *E. coli* (Smith and Huggins 1976; Couturier et al. 1988). ColV plasmids also contain an F transfer region and regions encoding plasmid maintenance and stability (Waters and Crosa 1991). Colicin has been referred to as bacteriocin, but has been grouped within the microcin class of antimicrobial polypeptides due to its small size and mechanism of synthesis which different from other colicins (Yang and Konisky 1984).

Genes for ColV of *E. coli* are in an operon which includes *cvaA*, *cvaB*, *cvaC*, *cvi* and *TolC* (Neidhardt and Curtiss 1996; Johnson et al. 2006). The *cva* genes are responsible for ColV production, export and immunity. There are *cvaA*, *B*, *C* for colicin production and *cvi* for ColV immunity protein which protect the cell from ColV bactericidal activity by encoding cognate immunity proteins and transmembrane helicases that efficiently protect the cell from bactericidal activity of ColV (Gerard, Pradel, and Wu 2005). Whereas, the *cvaA*, *cvaB* and *tolC* genes are responsible for ColV export through an ABC exporter with a requirement of membrane integrity, the *cvaC* encodes the colicin (Gilson, Mahanty, and Kolter 1987; Gerard, Pradel, and Wu 2005). In addition, the chromosomally encoded, *cvp,* is required for ColV production; however its function is unknown (Fath, Mahanty, and Kolter 1989; Gerard, Pradel, and Wu 2005).

Bactericidal activity of ColV is caused by disrupting of the membrane when ColV access to the inner membrane and attach to specific inner membrane receptor called the SdaC (DcrA) protein (Gerard, Pradel, and Wu 2005). Some studies revealed that the virulence phenotype conferred by ColV plasmid might resulted from other traits produced by the plasmid rather than the colicin V itself (Wick et al. 2005; Quackenbush and Falkow 1979). These include iron acquisition mechanisms and serum resistance. Iron acquisition was attributed to the presence of the aerobactin siderophore system, whereas; serum resistance was attributed to the presence of the *iss* and *traT*

genes which presented on ColV plasmids of APEC (Johnson et al. 2002). Apart from these two traits, ColV plasmid also have been associated with adherence, resistance to chlorine and disinfectants, growth in human urine, improved growth under acidic pH conditions, bacteriophage resistance, and the establishment of avian colibacillosis and murine septicaemia, meningitis and UTI (Hicks and Rowbury 1986; Aguero et al. 1989; Raja et al. 1991; Hu 1992; Provence and Curtiss 1994; Skyberg et al. 2006; Johnson and Nolan 2009).

Another ColV-related plasmid is ColBM plasmids which have evolved from ColV plasmids and encode for colicins B and M, discovered in APEC strain and MDR *E. coli* strain (Johnson, Johnson, and Nolan 2006; Tivendale et al. 2009; Fricke et al. 2008). There were reports that ColV and ColBM plasmids have acquired MDR-encoding regions, conferring resistance to antimicrobial agents such as tetracycline, ampicillin, streptomycin, and trimethoprim (Fricke et al. 2009; Fricke et al. 2008). The linkage between MDR-encoding regions and ColV-encoded PAI is beneficial for selection of highly virulent strains through the use of antibiotics (Johnson and Nolan 2009).

1.3.2 Haemolysin

Haemolysin is a calcium dependent, pore forming cytolysin of the repeats in toxin (RTX)-family. The cytolytic activity of *E. coli* haemolysin is attributed to rapid formation of cation-selective channels in host cells, leading to osmotic lysis (Beutin et al. 1989; Welch 1991). There are two types of haemolysin; α-haemolysin and enterohaemolysin (Burgos and Beutin 2010). Haemolytic activity of α-haemolysin causes big clear zones of haemolysis after 4-6 hours of incubation (during growth phase) at 37˚C on standard blood agar plates (Beutin et al. 1990; Schmidt, Beutin, and Karch 1995). Whereas, enterohaemolysin is recognised by the appearance of small turbid zones of haemolysis without inner clear zone on blood agar plates with phosphate-buffered saline (PBS)-washed erythrocytes after 18-24 hours of incubation at 37˚C (Beutin et al. 1989; Schmidt, Beutin, and Karch 1995). Both types of haemolysin are encoded by four genes within *hlyCABD* operon, found on either the chromosome or plasmid which bacteria can acquire through horizontal gene transfer. Conserve regions of the *hlyCABD* gene operon consist of regulatory gene (*hlyR*) located upstream of the operon and IS*911* element located downstream of *hlyD* gene. The presence of IS-elements flanking the plasmid-encoded *α-hly* indicate that they might be mobile genetic elements.

The plasmid-encoded *α-hly*, such as pEO5, pHly152 and pEO857, is associated with ETEC, STEC (Shiga toxin-producing *E. coli*) and EPEC strains. Whereas, *α*-haemolysin found in UPEC strains is encoded on chromosomal PAI I and II, such as strain O6:K15:H31 and O4:K6 found in human UPEC (Burgos and Beutin 2010; Burgos et al. 2009). Plasmid-inherited *α-hly* have evolved from one origin and independently from the chromosomally haemolysin operons because; 1) Regulatory region of *hlyC* of plasmid encoded *α-hly* genes were different from chromosomally inherited *α-hly* and 2) The 5' regulatory *hlyR* regions between chromosomally and plasmid-inherited *α-hly* operons are different that the chromosomally-encoded *α-hly* operons lack the *hlyR* and IS*911* elements (Burgos and Beutin 2010; Burgos et al. 2009). Alpha-haemolysin is synthesised as an inactive polypeptide and converted into active form by the HlyC protein, then secreted through the mediation of specific membrane translocator system encoded by *hlyB* and *hlyD* with the assist of outer membrane protein TolC (Schmidt, Beutin, and Karch 1995). The size of *α-hly* plasmids ranges from 48-157 kb. Not all the *α-hly* plasmid are conjugative, for example, pHly152 is a non-conjugative plasmid found in murine *E. coli* strain, whereas pEO5 found in human EPEC is conjugative (Burgos et al. 2009; Hess, Pyper, and Clements 1986). Most of the *α-hly* plasmids have evolved from a common origin (Burgos and Beutin 2010).

Another type of haemolysin is enterohaemolysin or EHEC-haemolysin which encoded on plasmid O157 (pO157) which is an F-like 92-kb plasmid within Inc group FIB and FII, found in EHEC strain such as *E. coli* O157:H7, O26 and O111. These strains are included in Shiga toxin-producing *E. coli* (STEC) that causes HC and HUS in humans (Carattoli et al. 2005; Johnson and Nolan 2009; Lim et al. 2007; Schmidt, Kernbach, and Karch 1996). The plasmid has the closest genomic sequence with the *Yersinia pestis* chromosome and plasmid, implying that pO157 was acquired from *Y. pestis* (Hinnebusch et al. 2002; Johnson and Nolan 2009). Enterohaemolysin is serologically and genetically different from *α*-haemolysin since a monoclonal antibody to *E. coli* *α*-haemolysin does not bind to enterohaemolysin. In addition, DNA of enterohaemolysin-producing *E. coli* does not hybridise with an *α*-haemolysin-specific gene probe (Beutin et al. 1990). Biologically active enterohaemolysin was detected only in bacteria grown to the stationary phase and the haemolysin did not released into the culture medium (Beutin et al. 1990). Apart from Shiga toxins (Stx) and the locus of enterocyte effacement (LEE) pathogenicity island, the EHEC haemolysin is also responsible for the enterohaemolytic phenotype conferring adherence ability to

intestinal epithelial cells and long-term colonisation, and cellular damage of *E. coli* O157:H7 as well as survival in vivo and in the environment (Lim et al. 2007; Welch 1991). There are several other putative virulence factors encoded by pO157, including enterohaemolysin (*ehxA*), general secretory pathway (*etpC* to *etpO*), serine protease (*espP*), catalase-peroxidase (*katP*), a potential adhesion (*toxB*), a Cl esterase inhibitor (*stcE*) and attaching and effacing gene-positive conserved fragments (*ecf*) (Lim et al. 2007). Enterohaemolysin was found to be closely associated with verotoxin (Shiga-like toxin) production of verotoxin-producing *E. coli* (VTEC) strains in pigs and cows both healthy and with diarrhoea. Around 95% of the VT^+ *E. coli* can produce enterohaemolysin, while only 5% of VT^+ *E. coli* was found to produce plasmid-encoded α-haemolysin (Beutin et al. 1989).

1.3.3 Enterotoxin

Enterotoxin is a plasmid-encoded virulence factor in ETEC strains causing self-limiting watery diarrhoea in human, and neotal and post-weaning pigs (Johnson and Nolan 2009). ETEC strains can produce either heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), or both which most of the strains also express fimbrial adhesins known as colonisation factors (CFs). The plasmids encoding enterotoxin and CFs in human contain different regions of FII Inc group; replication, transfer and stability regions (Johnson and Nolan 2009; Gyles 2004). In porcine ETEC strains, genes encoding enterotoxins and CFs were found on separate plasmids and the plasmid encoding F18 CFs also contains haemolysin determinant and replicon of IncFII and IC (Fekete et al. 2002; Johnson and Nolan 2009). One plasmid, containing a PAI with a fragment of genes encoding STs known as pTC also contains tetracycline resistance (Fekete et al. 2003; Olasz et al. 2005). Some of the transfer regions are incomplete; therefore, the plasmids require other plasmids for mobilisation and co-transfer (Johnson and Nolan 2009; Reis et al. 1980).

The LT is an immunogenic protein which is antigenically related to cholera toxin, affecting the adenylate cyclase system by deregulation. This results in over-production of cyclic adenosine monophosphate (cAMP), leading to opening of chloride channels in crypt cells and blockage of NaCl adsorption in apical tip cells which cause loss of water and electrolytes into the intestinal lumen, resulting in diarrhoea, hypovolemia and metabolic acidosis (Gyles 2004). There are two

serological subclasses of LT encoded on chromosome; 1) LT I, found in humans (LTh-I) and swine (LTp-I), 2) LT II (Hirsh and Zee 1999; Mainil 2013).

The ST is non-immunogenic protein which affects the guanylate cyclase system within the cell by deregulating cGMP synthesis (Hirsh and Zee 1999). There are two types of ST; STa and STb. The STa can be encoded either by the genes located on plasmid or a transposable element (transposon), causing fluid and electrolyte accumulation in the intestines of suckling piglets by blockage of NaCl and water adsorption in tip cells, and loss of Cl ion at crypt cells. The STb is an uncommon plasmid-encoded toxin, causing fluid accumulation in piglets and weaned pigs (Hirsh and Zee 1999; Mainil 2013).

1.3.4 Capsular pilus antigen (K88)

The K88 fimbriae or F4 antigen was first discovered in 1961 as the first colonization factor discovered on the cell surface of porcine ETEC strains, conferring ability to adhere to the epithelial mucosa of the small intestine and causing watery diarrhoea, dehydration and death in piglets (Nagy and Fekete 2005; Orskov et al. 1961; Fairbrother, Nadeau, and Gyles 2005; Jones and Rutter 1972). The adhesion is essential for the virulence of K88-containing bacteria by allowing the bacteria to attach to epithelium and overcome gut motility of the small intestine (Jones and Rutter 1972). Colonisation characteristics of the K88-positive and the K88-negative were not different. The K88-positive strains were presented at the tissue surface, mostly adhere to the tips and the sides of villi, and epithelial mucous; however, absent from the intestinal lumen. Most of the K88-positive strains were present in posterior small intestine. While, some strains were located in anterior small intestine and in the middle of small intestine. These strains also produced K88 antigen in the large intestine but did not adhere to the epithelial surface of the large intestine or penetrate into the crypts of Lieberkühn. Unlike the K88-positive strains, the K88-negative strains were evenly distributed throughout the intestinal lumen and not attached to the tissue surface. Both K88-postive and K88-negative strains cause clinical disease to piglets; however, severity of disease and mortality rate of the K88-negative strains were lower than those of the K88-positive strains (Jones and Rutter 1972). It was also associated with enteritis and oedema disease in swine (Nagy and Fekete 2005). In addition, there are other key virulence factors that have been implicated in ETEC causing postweaning diarrhoea such as haemolysins and F18 fimbriae (DebRoy and Maddox 2001). The

K88$^+$ ETEC strains belong primarily to the O8, O45, O138, O141, O147, O149, and O157 serogroups (Garcillan-Barcia, Francia, and de la Cruz 2009).

K88 is encoded by a transmissible virulence plasmid (K88$^+$ plasmid) which classified into three genetic variants based on antigenic regions, K88ab, K88ac and K88ad (Orskov and Orskov 1966; Fairbrother, Nadeau, and Gyles 2005). The K88ac is the most common variant among porcine ETEC strains (Fairbrother, Nadeau, and Gyles 2005; Nagy and Fekete 2005). The K88ab and K88ac operons appear to be located on a RepFIIA plasmid backbone. These variants bind to carbohydrates or glycoconjugants present on intestinal epithelial cells, intestinal mucus or red blood cells with different porcine cell preferences (Huisman et al. 1994). There are ten different genes for the operon encoding the K88, *faeABCDEFGHIJ* which divided into regulatory genes (a major subunit, FaeG and minor subunits, *faeCFHIJ*) and chaperone genes (*faeDE*), surrounded by insertion sequences which mediate virulence of the plasmid (Huisman et al. 1994). Many K88 plasmid-containing isolates were found to have an F-type replicon and some possess conjugation systems resembled to those of the IncI1 incompatibility group, implicating the possibility that K88$^+$ plasmids might belong to either the IncF or IncI1 group (Bradley 1985; Mainil et al. 1998).

1.4 Use of alternatives to antibiotics to treat *E. coli* infection

There are several approaches that have been implemented to prevent or delay AMR and to target virulence factors of the microbes using preventive and therapeutic measures. Vaccine is commonly used as preventive measure for bacterial infection. Since AMR has been increasing, the use of antibiotics is more restricted and must be used judiciously. Combination of antibiotics of different targets and recycling of antibiotics are also applied to delay the AMR (Gaur 2017). Therapeutic alternatives to antibiotics were categorised into naturally occurring, synthetic, and biotechnology-based alternatives (Ghosh et al. 2019). Bacteriophages have been used as alternatives in forms of phage therapy and genetically modified phages. Another interesting strategy is bacterial virulence factor inhibitors which neutralise or inhibit expression of virulence factors through the uses of specific antibodies and liposome-based virulence inhibitor (Ghosh et al. 2019).

Sex pilus-specific RNA bacteriophage, MS2, was found to reduce the carriage of AMR plasmids encoded by conjugative F-like plasmids by causing the loss of plasmids and blocking bacterial conjugation (Davis and Sinsheimer 1963; Colom et al. 2019). Several F-like plasmids also encode

virulence factors such as colicin V, enterohaemolysin, enterotoxin and fimbriae (Carattoli et al. 2005; Couturier et al. 1988; Huisman et al. 1994; Johnson and Nolan 2009; Smith and Huggins 1976). It is more likely that sex pilus-specific phage would affect expression and function of these virulence factors. Therefore, study of interaction between sex-pilus phage and its pathogenic hosts is essential for better insight of another alternative to antibiotics.

1.5 Bacteriophage biology and applications

MS2 bacteriophage was used in this study. This icosahedral lytic phage is within the genus *Levivirus* of the family *Leviviridae.* Its RNA contains approximately 3,500 nucleotides coding for maturation protein, coat protein, lysis protein and RNA replicase (Calendar 2006). Growth characteristics of MS2 phage include burst size of 10±1 PFU/cell and latent period of 60±2 minutes (Jain et al. 2006). More than 80 percent of the phage was adsorbed within different amount of times, ranging from 5 to 14 minutes (Davis and Sinsheimer 1963; Colom et al. 2019)

There are two main genomic types of bacteriophages; DNA and RNA phages, either single-stranded (ss) or double-stranded (ds). Moreover, bacteriophages can be classified by their morphology, including filamentous, icosahedral, tailed and pleomorphic (Abedon 2008; Calendar 2006). Types of phage infection (Table 1.3) are determined by several criteria; bacterial survival, phage survival, phage genome replication, phage virion production, phage progeny release (Abedon 2008). Lytic phages reproduce within the bacterial hosts and kill the hosts by lysis, then their progeny is released from the damaged cells. Phages undergoing both lysogenic and lytic cycle, namely temperate phages, are less virulent than the lytic phages (Abedon 2008).

Table 1.3 Stages of phage infection and mechanisms (Abedon 2008)

Stages of phage infection	Mechanisms
Lytic	Bacterial host die from full reproduction of phages
Lysogenic	Non-productive stage, but phage genome can replicate or integrate into the host chromosome to produce the prophage which is inheritable
Chronic	Continuous phage production with the survived bacterial host
Restriction	Bacteria are immunized to infection or adsorbing phages
Resistance	Absence of irreversible adsorption, both phage and host survive

Process of phage infection initiates with attachment of phage to its host, followed by conformation changes of host cell membrane, and DNA injection into the host cytoplasm. Phages adsorb (attach) and infect their hosts through interaction between phage receptor-binding proteins (RPBs) and bacterial cell surface receptors, conferring binding specificity of phages and determining their bacterial host range. Gram-positive and -negative bacterial receptors can be either protein- or saccharide-based, including lipopolysaccharide (LPS), porins, peptidoglycan (PG), teichoic acids, pellicle layer, capsule, and protruding organelles (flagella, pilus) (Dunne et al. 2018; Nobrega et al. 2018). Tailed bacteriophages use their tail components such as tail fibres, tail spikes or tail tips to interact with host receptors (Nobrega et al. 2018). Whereas, some bacteriophages only adsorb to contractile pili such as pilus phages, including filamentous DNA phages and icosahedral single-stranded RNA phages (Clarke et al. 2008; Rakhuba et al. 2010). Attachment of phages initiates with reversible when phage RPBs recognise bacterial receptors. Following this, the phage RPBs bind to bacterial outer membrane and the attachment becomes irreversible which induces opening of the phage connector and releasing of the genome into the bacterial cell (Kurtböke 2012). Single-stranded phages such as MS2, a sex pilus-specific ssRNA phage, infect Gram-negative bacteria by using a maturation protein (Mat), presented on the phage capsid together with coat proteins, to attach to a pilus of the host. When ssRNA phages adsorbs to the side of a pilus, the binding process induces small orientational changes of the Mat related to the phage capsid. The presence of a network of hydrophobic and electrostatic interaction at the phage-pilus interface is detected. This results in priming of the Mat-connected genomic RNA (gRNA) to be releasing from the phage. After being released from the capsid, Mat-connected gRNA is taken up by the host through the retractile force of the host pilus (Clarke et al. 2008). Then, expression of the viral genes has initiated by cytosolic internalisation of the gRNA, resulting in replication, virion morphogenesis and lysis of the host (Chamakura and Young 2019; Meng et al. 2019).

Bacterial hosts can develop resistance to phages. The MS2 phage infecting *E. coli* is capable of inducing phage-resistant mutants in the progeny of *E. coli* cells and selecting pre-existing phage-resistant mutants from mutations in the region of the F-factor in the bacterial chromosome as a result of interaction between phage RNA with bacterial DNA. The interaction leads to loss of F-pili and absence of RNA phage penetration into the host cell. MS2-induced mutants also exhibit

genetic instability and segregate new forms with new biochemical traits due to deletions of the region of the F-factor (Pererva, Miriuta, and Miriuta 2008).

Bacteriophages have been applied in phage expression systems, phage display, pollution indicators, diagnostic systems and phage therapy. Phage-based expression systems have been used to engineer bacterial plasmids for molecular biology study. Phage display provides huge libraries of peptides or proteins for study of protein-target interaction. Phages have been used to detect enteric pathogens in water as indicator organisms. As bacteriophages have narrow host range, they have been used in diagnosing several pathogens that cause diseases in human such as *Salmonella* and *Listeria*. However, this application has not yet commercially succeeded due to development of phage resistance. Phages also have been used in treatment of infectious diseases as alternative antibacterial therapeutic agents to antibiotics (Calendar 2006).

1.6 Aims

The aim of this study was to explore the potential of sex pilus-specific phages to drive loss and transfer of virulence plasmids from *E. coli*.

The key objectives were to:

1. Assess existing pilus target phages for infection of virulence plasmid containing strains.
2. To determine the natural transfer rate of the virulence (pHaemolysin and pColicin V) and F plasmids.
3. To determine the impact of phage infection on the virulence plasmids.

2 Materials and methods

2.1 Bacterial and bacteriophage strains

Fifteen *E. coli* strains containing different plasmids were selected for this project (Table 2.1). Control strain *E. coli* K12/*pFlac::*Tn*3*, *a* lactose-fermenting strain containing F plasmid tagged with Tn3 (Amp^R), was used as a host for MS2 bacteriophage replication (Colom et al. 2019; Davis, Sinsheimer, and Strauss 1961). Phage MS2, a ssRNA sex pilus-specific bacteriophage was used in this study.

Table 2.1 *E. coli* strains used in the experiment

***E. coli* strain**	**Plasmid contained**
Proto/*phly* amp^R	Haemolysin, ampicillin resistance
H209/*phly*	Haemolysin
H209 nal^R	Nalidixic acid resistance
F157/*pcolv*	Colicin V
H247/*pcolv*	Colicin V
H247	Putative colicin V
H209	Putative haemolysin
Proto/*phly*	Haemolysin
K12 nal^R	Nalidixic acid resistance
H209/*phly*	Haemolysin
K12/*pcolv*	Colicin V
711 nal^R	Nalidixic acid resistance
P123/*phly*	Haemolysin
K12/*pFlac::*Tn*3*	Fertility tagged with ampicillin resistance
J62 rif^R	Rifampicin resistance

2.2 Growth and culture of bacteria

All bacteria were preserved in 50% glycerol (1:1) and stored in -20˚C freezer until use. Luria-Bertani (LB) broth and agar (Oxoid, UK) were used for routine growth and maintenance of bacteria. MacConkey agar (Oxoid, UK) was used to distinguish lactose-fermenting *E. coli* strains.

To assess haemolysis, Sheep blood agar (SBA) was used. SBA was made by adding sheep blood at 5% v/v (Oxoid) in LB agar (Oxoid, UK).

Top agar (overlay agar) was used for colicin V activity test and phage count. The agar was made of LB broth (Oxoid, UK) and 0.7% w/v of select agar (Oxoid, UK). The top agar was sterilised

and cooled to 50°C prior to adding bacteria for use as overlay for colicin V test and phage count. To assess colicin production, a colicin sensitive strain (*E. coli* K12 *nal*^{R}) was used in an overlay agar with the ratio 1 : 0.1 (top agar : LB broth overnight culture), total volume of approximately 3 ml for 99 mm Petri Dish. For phage count, phage sensitive *E. coli* strain K12/*pFlac::*Tn*3* was used with the ratio 1 : 0.04 (top agar : LB broth overnight culture) of the total volume as same as the colicin V test. The top agar mixture was evenly poured on the 20-ml LB agar plate to make bacterial lawn and left to dry before adding samples.

All media were made according to manufactures diluted with sterile distilled water (DW) and autoclaved. Sterile PBS (Sigma-Aldrich, UK) was prepared following manufactures instructions in DW and sterilised by autoclave.

2.3 Purified MS2 bacteriophage preparation

Phage MS2 plaque formation and PFU assessments were assessed on *E. coli* strain K12/*pFlac::*Tn*3*. To ensure that *E. coli* strain K12/*pFlac::*Tn*3* was plasmid positive, the bacteria were recovered on MacConkey agar and incubated aerobically overnight at 37˚C. A single lactose-fermenting colony was used to inoculate 10 ml LB broth and incubated aerobically overnight at 37˚C prior to use.

To culture phage MS2, 1 ml of overnight K12/*pFlac::*Tn*3* was used to inoculate 9 ml of LB broth and 100 µl of MS2 phages from -20˚C were added. This was then incubated in a shaking incubator at 37˚C, overnight.

To purify MS2, the overnight phage-*E. coli* culture was transferred to 25-ml Falcon tube and centrifuged 6,100 g for 15 minutes at room temperature (ca. 20˚C), the supernatant fluid was then filtered through 0.45 µm nylon filter to remove residual bacteria. Five hundred microlitres of purified MS2 phage was mixed with 500 µl of 50% glycerol to make a fresh glycerol stock and stored at -20˚C, while the remaining phages were stored at 4˚C and used as a working stock.

2.4 Phage count

To quantify plaque forming unit per ml (pfu/ml), an overnight culture of *E. coli* strain K12/*pFlac::*Tn*3* was used to prepare a bacterial overlay plate. Decimal dilutions of MS2 were prepared to 10^{-8} in PBS. Ten microlitres of each MS2 dilution were places on the top agar and allowed to dry in laminar flow cabinet. Plates were then incubated aerobically at 37˚C overnight. Finally, the number of plaques of the most disseminated and clear dilution factor was counted, and then calculated for pfu/ml by using the following formula;

$$\text{pfu/ml} = \frac{\text{number of plaque}}{\text{dilution} \times \text{volume of inoculated phage}}$$

number of plaques = number of plaques of the most disseminated and clear dilution factor, dilution = dilution which the plaques are counted, volume of inoculated phage = 10 µl or 0.01 ml

2.5 Haemolysin activity test

E. coli strains were tested for haemolysin activity by streaking bacterial overnight LB broth culture on 5% v/v sheep blood agar and incubate in 37˚C incubator, overnight. Then, haemolysis zone were observed among the tested strains.

2.6 Colicin V activity test

E. coli strains were tested for colicin V activity by streaking or spotting single colony of each strain on LB agar plate topped with agar overlay of colicin V susceptible *E. coli* strain K12 nal^R and incubated at 37˚C overnight. The plates were observed for clear zone around the colony indicating colicin V activity.

2.7 Phage multiplication on selected *E. coli* strains

Single colony of *E. coli* strains, including K12/*pFlac::*Tn*3*, picked up from LB and MacConkey agars, respectively, were inoculated into 10 ml of LB broth. Next, the overnight cultures in 10-ml LB broth were diluted at ratio 1:100 and incubated in shaking incubator at 37˚C for 2 to 3 hours to reach mid-exponential phase with bacterial concentration of approximately 10^7 cfu/ml. At the same

time, MS2 phage stock was diluted with sterile PBS to concentration 10^6 pfu/ml and then 100 µl dilute phage was transferred to the diluted LB broth cultures so that phage concentration will be 10^5 pfu/ml. Before incubating in shaking incubator at 37˚C for 24 hours, phage count at 0 hour was performed by taking 1ml aliquot of each *E. coli*-phage mixture and filtering through 0.45 µm nylon. Then, the aliquot samples were diluted from dilution -1 to -4 with sterile PBS (ratio 1 : 9, total volume 200 µl). Negative control was also included by diluting MS2 phage 10^6 pfu/ml with 10-ml LB broth to reach final concentration 10^5 pfu/ml. Top agar (20 ml) plates were prepared with 120-µl *coli* K12/*pFlac::*Tn*3* and divided into 4-dilution compartments. After that, 10 µl of each diluted aliquot was dropped on Top agar according to their dilution factor from dilution -4 to -1, the plates were left to dry for a while and then transferred to 37˚C incubator and incubated overnight. Overnight LB broth culture of *E. coli* K12/*pFlac::*Tn*3* was also prepared from a single lactose-fermenting colony with 10 ml of LB broth and incubated in shaking incubator at 37˚C for making bacterial overlay at 24-hour phage count the next day.

Plates of phage count at 0 hour were observed for plaque number and calculated for pfu/ml. After 24 hours of *E. coli*-phage mixture incubating, 1-ml aliquot of each mixture was taken and filtered through 0.45 µm nylon. Phage count at 24 hours was performed with the same process as the same as 0-hour phage count, the only exception is that the 24-hour aliquots were diluted from dilution -1 to -8 and phage count was performed from dilution -3 to -8 pfu/ml was calculated by using the formula mentioned in section 2.4.

If there were no plaque, use zero plaque calculation;

$$\frac{\text{pfu}}{\text{ml}} = \text{lowest dilution} \times \text{number of plaque} \times \text{factor volume for aliquot}$$

Lowest dilution = the most concentrated dilution of a particular plate, number of plaques = 1, factor volume = incubated phage amount made up to 1 ml which is 10 µl plus 100 to make 1 ml

2.8 Attachment of phage to selected *E. coli* strains

All test strains were prepared in LB agar and aerobically incubated at 37˚C, also the *E. coli* K12/*pFlac::*Tn*3* was prepared in MacConkey agar with the same conditions. A single colony of

each strain was picked up and inoculated in LB broth 10 ml and incubated in shaking incubator at 37˚C overnight (12h). The overnight cultures were diluted at ratio 1:100 with 10-ml LB broth and incubated in shaking incubator at 37˚C for 2-3h. Then, MS2 phage stock was diluted with sterile PBS to reach final concentration 10^7 pfu/ml (MOI 0.1) by diluting phage stock from initial concentration to 10^8 pfu/ml. Inoculated phage volume was calculated by using the following formula;

$$\text{MOI} = \frac{\text{pfu} \times \text{inoculated phage volume}}{\text{cfu} \times \text{broth culture volume}}$$

With the given MOI of 0.1, concentration of inoculated bacteria and phage was 10^7 cfu/ml and 10^8 pfu/ml, respectively, then the inoculated phage volume would be 0.1 ml or 100 µl in 10-ml broth culture. After inoculating, the mixture was mixed by inversion 2-3 times. Negative control was also prepared by mixing 10 ml of sterile PBS and 100 µl of 10^8 pfu/ml phage and followed the same process as the tested strains. All tested mixtures and negative control were incubated in shaking incubator at 37˚C for 10 minutes, then 1 ml of each sample was taken up and filtered through 0.45 µm filter immediately. Overlay agar plates were prepared with K12/*pFlac::*Tn*3*. The plates were divided into 6 dilutions from -1 to -6. The filtered samples were diluted with sterile PBS from dilution -1 to -6 (20 µl of filtered sample: 180 µl of PBS), then 10 µl of each sample including negative control was dropped on Top agar plate according to their dilution and incubated in incubator at 37˚C, overnight. Finally, plaques were count and calculated for pfu/ml using the mentioned formulas above.

2.9 Determining lactose, colicin V and haemolysin plasmids transfer rate

This experiment was duplicated. The first attempt was achieved by static mating; whereas, the second attempt was done by filter mating to increased efficiency of conjugation. All donor strains and recipient strains were incubated overnight in 10-ml LB broth at 37˚C. Recipient and donor strains were inoculated into 10-ml of LB broth at ratio 5 : 1 and 1 : 1 for static mating (final volume 60 µl) and filter mating (final volume 100 µl), respectively. 50-µl aliquots of recipient strain and 10-µl of donor strain were inoculated into 10-ml of LB broth in static mating. A negative control was prepared with 50 µl of recipient alone in 10-ml LB broth. The mating mixture and control were incubated statically overnight at 37˚C for static mating. For filter mating, 100 µl 1 : 1 mixture

of donor and recipient were spread onto filter paper (Whatman, UK) and placed on LB agar plate and incubated statically overnight at 37˚C. After incubation the mating mixtures from static mating were diluted with LB broth from approximately 10^9 cfu/ml to 10^2 cfu/ml and 300 µl of diluted mating mixtures were plated on LB agar with antibiotic. Whereas, each incubated filter paper was transferred to a tube with 1-2 ml PBS and vortexed to remove bacteria from the paper, then diluted from dilution -1 to -6. The diluted samples were tested for single colony by dropping 10 µl of each dilution on LB agar with rifampicin 20 µg/ml. The dilutions giving single colony were then diluted with LB broth at ratio 1 : 9 and 300 µl of each diluted sample was spread on LB agar plate containing rifampicin 20 µg/ml. Except for K12/*pFlac::*Tn*3*, LB agar plates with 20 µg/ml rifampicin, 100 µg/ml ampicillin, and 20 µg/ml rifampicin with 100 µg/ml ampicillin were also applied for this strain.

At least one hundred colonies were required for each strain to detect lactose fermentation, haemolysin and colicin V phenotypes. MacConkey agar, LB agar with K12 *nal*^{R}, and 5% blood agar without antibiotic were prepared for lactose, colicin V and haemolysin plasmids detection, respectively. A plate was divided into at least 50 squares, so two plates were required to contain 100 colonies for each sample. Following this, 100 colonies of each sample were picked and spot on particular agar according to the plasmid they carried, lactose plasmid-containing recipient was transferred to MacConkey agar, colicin V plasmid-containing recipient was transferred to LB agar with K12 *nal*^{R} lawn, and haemolysin plasmid-containing recipient was transferred to blood agar. All plates were incubated at 37˚C overnight. Finally, lactose fermentation, colicin V and haemolysin activities were assessed.

2.10 Plasmids preparation

F, haemolysin, and colicin V plasmids were obtained for sequencing from *E. coli* K12/*pFlac::*Tn*3*, P123/*phly*, and H247/*pcolv*, respectively. QIAGEN Plasmid Midi kit (QIAGEN, UK) was used according to their sizes and copy numbers (Table 2.2), following the protocol for low-copy plasmids of 100 ml culture volumes to purify the plasmids (QIAGEN 2012). Agarose gel analysis was performed with the eluate products to determine the purification procedure that the products contain only plasmid DNA without contamination of other nucleic acids (Sigma-Aldrich, UK;

condition 95V, 90 minutes) (Sigma-Aldrich 2018). Plasmid DNA yields were also determined by NanoDrop™ (Thermofisher, UK) at 260 nm.

Table 2.2 Sizes and copy numbers of F, ColV and haemolysin plasmids

Plasmid name	Size (kb)	Copy number (copies)	Reference
F	ca. 100	1-2	(Rosenberg and Hastings 2001)
ColV	ca. 40	1	(Cascales et al. 2007)
pO157	92	3-4	(Lim et al. 2007; Noegel, Rdest, and Goebel 1981)

2.11 Statistical analysis

Descriptive statistic was used to analyse data obtained from the experiments.

3 Results and discussion

3.1 *E. coli* strain characteristics and MS2 phage count

To confirm existence of the virulence plasmids in the *E. coli* strains used in this study, thirteen *E. coli* strains were tested for haemolysin and colicin V activities on specific agars. The selected strains contain none to maximum one plasmid. Six strains were haemolytic *E. coli.* Whereas, there are only three strains that were positive for colicin V activity (Table 3.1). Those that contained virulence plasmids will be subjected to experiment with phage MS2 to test whether the phage can eliminate virulence characteristics of the *E. coli* strains.

Eight decimal dilutions of MS2 phage stock was count on susceptible bacterial lawn using overlay agar technique. The MS2 used in this study had concentration of $2.1*10^{11}$ pfu/ml. The stock was adjusted to optimal concentrations for each experiment.

Table 3.1 Haemolysin and colicin V characteristics of *E. coli* strains

***E. coli* strains**	**Haemolysin**	**Colicin V**
Proto/*phly amp*^{R}	+	–
H209/*phly*	+	–
H209 *nal*^{R}	+	–
F157/*pcolv*	–	+
H247	–	–
H209	–	–
Proto/*phly*	+	–

K12 *nal*^{R}	–	–
H209/*phly*	+	–
H247/*pcolv*	–	+
711 *nal*^{R}	–	–
K12/*pcolv*	–	+
P123/*phly*	+	–

+ = positive, – = negative

3.2 Attachment of MS2 phage

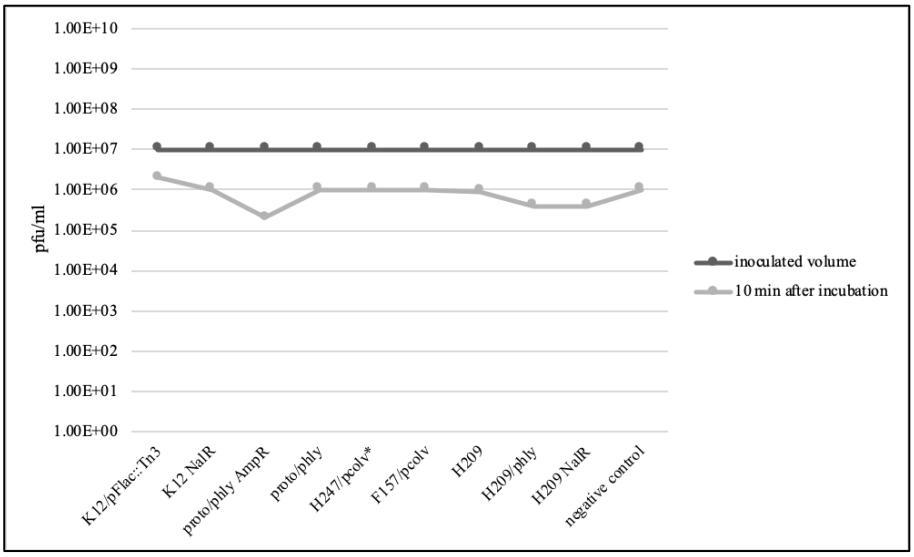
Phage attachment or adsorption experiment was accomplished to determine adsorption rate of the MS2 with *E. coli* strains in this study. Plaque forming unit per ml of free MS2 phage in supernatant after 10 minutes of incubation with tested *E. coli* strains decreased by 5 to 50 folds (average 11 folds) from an initial concentration 10^7 pfu/ml to the range of $2*10^5$ to $2*10^6$ pfu/ml (Table 3.2, figure 3.1). The highest decreased pfu/ml was in *E. coli* proto/*phly amp*^{R}. The lowest decreased pfu/ml was in K12/*pFlac::*Tn*3*. Whereas, the pfu/ml in negative control decreased by 10 folds. Decline in phage concentration in culture medium can be assumed as a result from phage attachment to the bacterial host. Percent of phage adsorption to host cell within 10 minutes was calculated using data obtained from the experiment, ranging between 10 and 24%. The highest and the lowest adsorption was in the strains proto/*phly amp*^{R} and K12/*pFlac::*Tn*3*, respectively. The results contradicted to the facts that K12/*pFlac::*Tn*3* was the susceptible host of MS2 phage and there should be no phage adsorption in negative control. However, the phage attachment experiment should be repeated because MS2 adsorption rate could not be determined from the preliminary experiment.

Table 3.2 Pfu/ml of MS2 measured in culture medium after attachment period with tested *E. coli* strains and percent of phage adsorption from a preliminary experiment

E. coli strains	**Phage count in supernatant (pfu/ml)**	**Phage attached to host cell (pfu/ml)**	**Percent of phage adsorption (%)**
PBS control	$1*10^6$	$1*10^1$	14
K12/*pFlac::*Tn*3*	$2*10^6$	5	10
K12 *nal*^{R}	$1*10^6$	$1*10^1$	14
Proto/*phly amp*^{R}	$2*10^5$	$5*10^1$	24
Proto/*phly*	$1*10^6$	$1*10^1$	14
H247	$1*10^6$	$1*10^1$	14
F157/*pcolv*	$1*10^6$	$1*10^1$	14
H209	$9*10^5$	$1.11*10^1$	15

H209/*phly*	$4*10^5$	$2.5*10^1$	20
H209 *nal*^{R}	$4*10^5$	$2.5*10^1$	20

Figure 3.1 Changes in phage concentration in culture medium after 10-minute incubation of *E. coli* strains with the phage MS2, assuming MS2 attachment to the *E. coli* strains. Repeat of this experiment is suggested due to a decrease of the phage in negative control.



3.3 MS2 host range

Phage host range experiment was quadruplicated to determine susceptibility of the tested *E. coli* strains to the phage MS2. Data obtained from the first replicate was not completed and slightly different from others; therefore, only the data from replicate 2,3 and 4 was summarised using mean (SD). Phage multiplication in the susceptible host *E. coli* K12/*pFlac::*Tn*3* increased within the range of $2*10^4$ to 10^6 folds of average $1.03*10^5$ folds. Whereas, multiplication of MS2 in tested *E. coli* strains increased by 1 to 1,300 folds with mean (SD) ranged between 1.78 (0.7) to 223 (191.01) (Table 3.3 and 3.4). Comparing with the *E. coli* K12/*pFlac::*Tn*3*, *E. coli* strains tested in this study were not likely to be susceptible to the MS2; however, the data was varied dramatically due to repression of the F pilus which complicate the results of this experiment. Further study should be implemented to explore other susceptible virulent *E. coli* strains.

Table 3.3 Multiplication in pfu/ml of MS2 after incubation with tested *E. coli* strains

Number of replicates	*E. coli* strains	pfu/ml at 0 h	pfu/ml at 24 h	Difference (folds)
1	PBS control	$3*10^6$	-	-
	K12/*pFlac::*Tn*3*	$1*10^4$	$1*10^{10}$	10^6
	P123/*phly*	<100	$1*10^6$	10^4
	H247/*pcolv*	$2*10^5$	$1.3*10^6$	1,300
	H209/*pcolv*	$1*10^5$	<1	0.001
	K12/*pcolv*	$2*10^5$	<1	0.001
2	LB broth control	$2*10^6$	$6*10^6$	3
	K12/*pFlac::*Tn*3*	$2*10^6$	$8*10^8$	$4*10^4$
	P123/*phly*	$3*10^6$	$2*10^7$	6.67
	H247/*pcolv*	$4*10^6$	$1*10^7$	2.5
	H209/*pcolv*	$4*10^6$	$2*10^7$	5
	K12/*pcolv*	$3*10^6$	$3*10^6$	1
3	LB broth control	$1*10^6$	$1*10^6$	1
	K12/*pFlac::*Tn*3*	$1*10^6$	$2*10^{10}$	$2*10^4$
	P123/*phly*	$6*10^5$	$2*10^7$	33.33
	H247/*pcolv*	$3*10^5$	$1*10^8$	333.33
	H209/*pcolv*	$2*10^6$	$2*10^6$	1
	K12/*pcolv*	$1.7*10^6$	$4*10^6$	2.35
4	LB broth control	$1*10^5$	$1*10^5$	1
	K12/*pFlac::*Tn*3*	$2*10^5$	$5*10^{10}$	$2.5*10^5$
	P123/*phly*	$1*10^4$	$2*10^6$	200
	H247/*pcolv*	$3*10^4$	$1*10^7$	333.33
	H209/*pcolv*	$1*10^4$	<1	0.0001
	K12/*pcolv*	$1*10^5$	$2*10^5$	2

Table 3.4 Mean (SD) of number of phage increased in pfu/ml of different *E. coli* strains

E. coli strains	Mean (SD) of increased pfu/ml
K12/*pFlac::*Tn*3*	$1.03*10^5$ ($1.3*10^5$)
P123/*phly*	80 (104.77)
H247/*pcolv*	223 (191.01)
H209/*pcolv*	2 (2.65)
K12/*pcolv*	1.78 (0.7)

3.4 Plasmid transfer rates

Prior to experiment of using phage to inhibit virulence plasmid transfer, natural plasmid transfer rates of *pFlac*::Tn*3*, *pcolv* and *phly* were determined comparing between two different methods;

static mating and filter mating. Transfer rates of haemolysin and colicin V plasmids were 0 to 8% and 0%, respectively. Whereas, transfer rate of F plasmid ranged from 10 to 97%, conferring high transmissible characteristic of the F plasmid (Table 3.5).

Low natural transfer rate of the *pcolv* and *phly* may implicate experiment of phage effect on virulence plasmid transfer ; therefore, it is essential to scrutinise sequences and expressions of the virulence plasmids as well as of the F pili.

Table 3.5 Transfer rate of F, haemolysin and colicin V plasmids of tested *E. coli* strains

Donor strains	recipient	Antibiotic used	Transfer rate	
			Static mating	Filter mating
K12/*pFlac::*Tn*3*	J62 *rif*^{R}	Rif 20 µg/ml	60%	10%
	J62 *rif*^{R}	Rif 20 µg/ml and Amp 100 µg/ml	-	93%
	J62 *rif*^{R}	Amp 100 µg/ml	-	97%
P123/*phly*	J62 *rif*^{R}	Rif 20 µg/ml	0%	1%
H209/*phly*	J62 *rif*^{R}	Rif 20 µg/ml	0%	8%
H247/*pcolv*	J62 *rif*^{R}	Rif 20 µg/ml	0%	0%
K12/*pcolv*	J62 *rif*^{R}	Rif 20 µg/ml	0%	0%

Rif = rifampicin, Amp = ampicillin

3.5 Plasmid preparation

Due to low susceptibility of *E. coli* strains to MS2 phage, low transfer rate of the virulence plasmids and repression of the F pili, plasmid sequencing and mRNA expression level of the F pilus were planned to be accomplished to determine sequence and expression of the F pilus as well as incompatibility group of the three plasmids. To roughly determine plasmid sizes, prepare plasmids for sequencing and ensure purity of the products, all plasmids were purified and analysed with agarose gel electrophoresis and determined for DNA yield according to the low-copy plasmids preparation protocol. Concentrations of *pFlac*::Tn*3*, *pcolv* and *phly* were 150.4, 56.4 and 73.74 ng/µl, respectively. Sizes of the three plasmids were beyond 1 kb (Figure 1.2) which were corresponded to those references mentioned in the Table 2.2. The plasmid products were sent for sequencing and will be subjected to alignment and analysed for mutations.

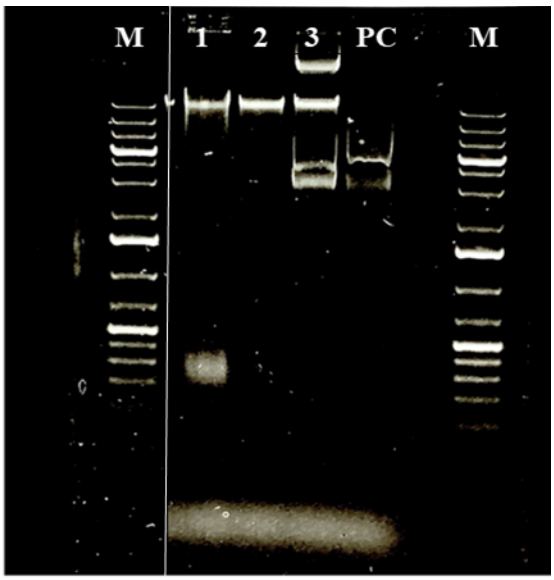


Figure 3.2 Agarose gel analysis of purified plasmids. Lanes are M = 1 kb plus ladder (Thermofisher, UK), PC = positive control; replicative M13 phage 7 kb, 1 = *pFlac*::Tn*3*, 2 = *pcolv*, 3 = *phly (trimmed to remove non-project lanes)*

3.6 Discussion

Due to COVID-19 pandemic, some experiments were put off and cannot be completed within the study period. This study was designed to examine virulence plasmid loss and reduction in frequency of conjugal transfer of virulence plasmids between *E. coli* strains through the use of sex pilus-specific bacteriophages. Bacteriophage susceptibility was assessed with *E. coli* strains harbouring certain virulence plasmids. Selected *E. coli* strains were characterised for bacteriophage susceptibility using the F pilus targeting phage MS2. Decreasing in the number of PFU in assays after 10-minute incubation is suggested the attachment of the phage to the *E. coli* strains but no replication. Comparing between *E. coli* strains with and without virulence plasmids, the *E. coli* strains containing non-virulence plasmids including the strains with AMR plasmids have phage attachment within the incubation period more than the strains with virulence plasmids and the phage susceptible strain. The K12/*pFlac::*Tn*3* susceptible to phage MS2 have the phage attached by half of the K12 with AMR plasmid. The proto strain containing virulence and AMR plasmids has phage attachment by 5 fold of the strain containing only *phly* plasmid. Comparing among the H209 strains, the strain without plasmid has phage attachment far less than the strains containing virulence and AMR plasmids. The strains containing *pcolv* plasmid have an equal amount of phage attachment. However, comparing all tested strains with the phage MS2

susceptible strain, the susceptible strain has phage attachment less than all tested strains, suggesting that the incubation time is not sufficient or there were factors interfering with the attachment. Due to a decreasing in the number of plaques in the negative control, and slight attachment of the phage to its specific host, K12/*pFlac::*Tn*3*, this experiment should be repeated, and incubation time should be extended. The extension of incubation period and replicating of experiment would provide better insight of the phage attachment to the *E. coli* strains.

Limited evidence of phage replication was observed between colicin V and haemolysin expressing *E. coli* strains, compared to the de-repressed MS2 host strain K12/*pFlac::*Tn*3*. There could be a reasons for this. The pili are not expressed or they are not receptive to MS2. Lack of expression of the F pili suggested repression of the transfer gene involving FinOP system, while lack of binding may be due to divergent sequences within the F pili producing genes (Frost and Paranchych 1988; Koraimann et al. 1996; Manchak, Anthony, and Frost 2002). Both these hypotheses would require further testing. Repression may be significant as the transfer rates for haemolytic and colicinogenic activity in this study are very low suggestive of a highly repressed transfer system. Repression of conjugative plasmid is probably to minimising the burden from either the host energy expended to maintain the conjugative apparatus or other associated properties such as pili-specific bacteriophage sensitivity (Ghigo 2001). However, the repressed conjugative plasmid can be temporarily de-repressed when the transconjugant acquires the plasmid, the newly transferred plasmid is transiently de-repressed until all the potential recipients have acquired the plasmid. Transitory de-repression often involves in biofilm formation to allow for horizontal plasmid transfer (Lundquist and Levin 1986). When plasmid transfer is completed, the conjugative pili recede and are replaced by adhesion factors such as other types of pili or exopolysaccharides in order to maintain the coherence of the biofilm (Ghigo 2001). In this work, phage infection was not assessed on biofilm forming bacteria so leading to a pilus repressed test population of bacteria. Therefore, one option to explore in further work would be to assess MS2 infection on model biofilms of these bacteria. This may be critical as expression during infection may be more akin to biofilms so F pilus may be de-repressed under infection conditions.

We propose that a better approach would be to determine the sequence of pili and use this data to develop tools to show expression of pilin. Moreover, plasmid sequencing would provide

information on mutations of the pilin genes within the pFlac, pHaemolysin and pColicin V plasmids that may impact on phage binding.

To determine mRNA expression levels of pilin from the plasmids in this study. We would determine the sequence for the *traA* gene responsible for F pilin production. Then design reverse transcriptase PCR primers to assess relative expression levels using SYBR green based qPCR. Pilin expression of the de-repressed pFlac pilin will be compared to pilin expression of the pHaemolysin and pColicin V plasmids. These expression levels will be standardised against *tufA* (encodes elongation factor Tu 1) and 16S RNA genes as controls. The proposed method will use primers specific to *traA* designed from sequence from our plasmids. Due to COVID-19 shut down this sequence was not obtained in time. Therefore, the *traA* nucleotide sequence from *E. coli* K12 plasmid F (accession number MK492260.1) was used as a model for primer design using Primer-Blast, NCBI. This uses a melting Tm of approximately 60°C and a GC-content of the primer pair is 55% (Table 3.6). The *traA* sequences of the pFlac, pHaemolysin and pColicin V plasmids would be subjected to the multiple sequence alignment program, Clustal Omega, (EMBL-EBI). This is to determine if homology is maintained across strains in this study. If there is significate sequence divergence then RT-primers would be designed individually for each plasmid. In addition to mRNA level of pilus, F Inc group typing can be determined from the sequence to confirm that the plasmids of the *E. coli* strains in this study are truly within the incompatibility group F.

Table 3.6 Primer pair specific to *traA* gene

Name	Primer sequence	Product size (bp)
F_traA	CGCCCGTCAAAAAGAAGTCG	282
R_traA	CAAAACCGGCCAGGAACTTG	

The lack of conjugal transfer of the pColicin V plasmids in the colicinogenic *E. coli* strains suggested that the plasmids were completely repressed, resulting in no expression of the F pili. Low transfer rate of the pHaemolysin plasmids suggested partial plasmid repression or combination of repressed and de-repressed plasmids. Whereas, the pFlac plasmid was entirely de-repressed, conferring the K12/*pFlac::*Tn*3* an ability to transfer its plasmid to the recipient. Taken together with the minor replications of the phage MS2 observed among the haemolytic and colicinogenic *E. coli* strains, this low expression levels of the F pili of pHaemolysin and pColicin V plasmids may explain the lack of MS2 replication.

Another possible cause of MS2 resistance is mutations of the transfer genes which affect expression of the F pili. However, there is a possibility that the phage could not recognise the expressed F pili. As described above, expression of the F pili can be confirmed by detection of F pili mRNA level. The exact coding of the pilin can also be assessed from the primary plasmid sequence to determine any polymorphisms. Alternatively, both expression of pilus and attachment of the phage to the pilus can be achieved by using electron microscopy as previously shown for MS2 (Meyvisch, Teuchy, and Van Montagu 1974).

Experiments on plasmid transfer rate in the presence of phage MS2 were planned to be accomplished in this study to determine the effects of the MS2 on virulence plasmid loss and horizontal virulence plasmid transfer. However, this could not be achieved due to the COVID-19 shut down. We hypothesised that in the presence of F pili, MS2 phage could eliminate the virulence plasmids and reduce virulence plasmids transfer. However, the infection, replication and conjugal transfer data would suggest this may not succeed.

Many research has been focused on using bacteriophage to target AMR and MDR bacteria, including reduction and prevention of AMR plasmids transfer, and AMR bacteria lysis (D'Accolti et al. 2019; Colom et al. 2019; D'Accolti et al. 2018). Although phage infection also contributed to transfer of AMR genes through transduction, the transduction rate was relatively lower than the conjugation rate (Volkova et al. 2014) This study is a novel aspect of using bacteriophage to mitigate the spread of virulence plasmids of virulent *E. coli*. Phages of several other pathogenic bacteria have been studied in reducing bacterial virulence as phage-based therapies. Some phage-resistant mutant bacteria were found to be less or non-virulent and cause less severe disease in their host. The absence virulence factors may be essential for phage attachment (Heierson et al. 1986; Laanto et al. 2012; Park et al. 2000; Santander and Robeson 2007). The phage-resistant bacteria can be beneficial for vaccine preparation against pathogenic bacteria as they lack of virulence factors but can modulate immune response and provide protection against pathogenic bacteria (Capparelli et al. 2010).

In conclusion, there may be limitations on use of F pili specific phage to target virulence plasmids. Phage susceptibility depends on expression of the transfer genes; therefore, de-repression of the F pili in virulent *E. coli* strains would be central to success of this approach. There are clearly

limitations to our data that we lack full evidence of pilus expression and phage binding to the specific pili. In addition, the data do not reflect environmental expression during infection where use of these phage may be critical.

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