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OTITIS MEDIA WITH EFFUSION:
CURRENT TREATMENT, NEW UNDERSTANDING OF
ITS AETIOPATHOGENESIS, AND A NOVEL
THERAPEUTIC APPROACH

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ABSTRACT

Otitis Media with Effusion (OME) is a common childhood condition leading to hearing loss, and its treatment with ventilation tubes (VTs) is one of the commonest surgical procedures. However, aetiology of OME is poorly understood, and its current treatment requires improvement as OME frequently recurs once VTs extrude.

The first, clinical part of this thesis showed that 63.6% of children randomised to VT insertion in a clinical trial will require VTs again, and even with additional adenoidectomy the need for repeat surgery remains high. Although published national guidelines set out criteria for surgery, the multicentre study presented here showed that only 32.2% of children that had VTs met these criteria, and guidelines' publication had limited impact on clinical practice.

The second, laboratory part of this thesis demonstrated the importance that bacteria and biofilms play in aetiology of OME, as live bacteria were demonstrated in 91.9% of middle ear effusions (using culture and confocal microscopy). Following from this, a Staphylococcus aureus biofilm model was developed, and used to show that biofilm eradication requires antibiotic (rifampicin and clindamycin) levels 1,000 times higher than those required to inhibit planktonic bacteria, over a period of 2-3 weeks. To achieve this in the middle ear, a local delivery strategy using biodegradable poly (lactic-co-glycolic acid) antibiotic pellets was proposed. Drug release from these pellets
was investigated with High Performance Liquid Chromatography and Serial Plate Transfer Testing, which demonstrated that antibiotics can be released for up to 3 weeks. Importantly, the pellets were able to eradicate biofilms in the *in vitro* model.

This thesis has shown that current OME treatment has significant deficiencies, but better understanding of OME pathogenesis raises the possibility of rational new therapeutic strategies. Biodegradable antibiotic pellets designed to eradicate OME biofilms may be a better future treatment that could improve the lives of countless children.
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**Internal Assessor:** Brigitte Scammell

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- Nottingham University Hospital Microbiology Department: Steve Holden
Serial plate transfer testing of antibiotic pellets was performed in conjunction with Husnah Hussein (doctoral student) and Rob Chessman (BMedSci / medical student), whose laboratory work was directed and supervised by myself. Shehla Imtiaz-Umer performed majority of the routine microbiology identification work on the middle ear effusion clinical samples; I performed some laboratory work on clinical samples, and then analysed the images, collated the data, and performed statistical analysis. The original TARGET data was collected by the TARGET multi-centre team, with my involvement dealing purely with the supplemental, long-term study in this thesis. With the exception of above acknowledgements, the thesis represents my own work.

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<td>+ad</td>
<td>plus adenoidectomy</td>
</tr>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
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<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CSOM</td>
<td>chronic suppurative otitis media</td>
</tr>
<tr>
<td>dB NHL</td>
<td>decibel Normal Hearing Level</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>ESZ</td>
<td>electrical sensing zone</td>
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<tr>
<td>FAO</td>
<td>further active observation</td>
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<tr>
<td>FISH</td>
<td>fluorescence In-Situ Hybridisation</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>ISA</td>
<td>Iso-Sensitest Agar</td>
</tr>
<tr>
<td>MBC</td>
<td>minimum bactericidal concentration</td>
</tr>
<tr>
<td>MBEC</td>
<td>minimum biofilm eradication concentration</td>
</tr>
<tr>
<td>ME</td>
<td>middle ear</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MR</td>
<td>modified release</td>
</tr>
<tr>
<td>NAC</td>
<td>N acetyl cysteine</td>
</tr>
<tr>
<td>NICE</td>
<td>Nice Institute of Clinical excellence</td>
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<td>OME</td>
<td>otitis media with effusion</td>
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<tr>
<td>OTT</td>
<td>obliged to treat</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PLG</td>
<td>poly(DL-lactide-co-glycolide)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>PV7</td>
<td>post-visit 7 study</td>
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<tr>
<td>RAOM</td>
<td>recurrent acute otitis media</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>RPC</td>
<td>reverse phase chromatography</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SPTT</td>
<td>serial plate transfer test</td>
</tr>
<tr>
<td>TARGET</td>
<td>trial of alternative regimens in glue ear treatment</td>
</tr>
<tr>
<td>TLT</td>
<td>TARGET long term study</td>
</tr>
<tr>
<td>TM</td>
<td>tympanic membrane</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone soya broth</td>
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<tr>
<td>VT</td>
<td>ventilation tube (tymanostomy tube, grommet)</td>
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Otitis media with effusion (OME, glue ear) is a common cause of hearing loss in childhood, affecting up to 80% of children at some stage at least for a limited time period. Treatment with grommets (ventilation tube, VT) insertion is one of the commonest surgical procedures in children in the developed world, and over 30,000 VTs are inserted in England each year. However, this treatment is far from ideal, as up to 25% of treated children have to have surgery more than once.

Despite its common nature many questions surround OME, and this thesis aims to address some of the current deficiencies in our knowledge of this condition and its treatment. In addition to laboratory-based research, the project also investigates a number of clinical aspects.

The clinical part of the thesis assesses recurrence after grommet extrusion, and details why further OME research is required. The role of adjuvant adenoidectomy in reducing need for revision surgery is examined. Current treatment is guided by the National Institute for Health and Clinical Excellence, and therefore actual clinical practice is compared with the guidelines, and the impact of guideline introduction assessed in a multi-centre project.

The main, laboratory-based, part of the thesis starts by consolidating the existing microbiology data obtained by our group, demonstrating a crucial role
for bacteria and biofilm in OME. Given the new understanding of OME pathogenesis and the limitations of current treatments, a novel therapeutic strategy is then explored. This centres on the use of high dose modified-release antibiotics, and this strategy is explored in a newly-developed *in vitro* model. A biodegradable antibiotic modified-release pellet is examined, assessing drug release with High Performance Liquid Chromatography and with Serial Plate Transfer Testing, and the effectiveness of the pellet against biofilms is tested in the *in vitro* model.
2 INTRODUCTION

2.1 OTITIS MEDIA WITH EFFUSION

Otitis media with effusion (OME, glue ear) is a common childhood condition. It is the commonest cause of deafness in children in the developed world with prevalence in the region of 20% (Mandel, et al. 2008), and up to 80% of children affected at least temporarily by the age of 10 years (Bluestone, Stephenson and Martin 1992). It is characterised clinically by hearing loss due to a middle ear effusion (thick fluid behind the ear drum) in the absence of symptoms or signs of acute inflammation, and pathologically by chronic inflammation of middle ear mucosa leading to the production of a mucin-rich effusion.

Although in majority of cases OME is transient, a proportion of children develop persistent symptoms that affect hearing, education, language or behaviour (Hall, Maw and Steer 2009) (NICE 2008). OME can also be a precursor to tympanic membrane (TM) retraction (TM becoming sucked into the middle ear), perforation, or cholesteatoma (skin in the middle ear). If OME persists after a three month period of watchful waiting and has a significant impact on the child, treatment with ventilation tubes (VTs, grommets) may be considered (Lous, et al. 2005) (NICE 2008). VT insertion is one of the commonest indications for surgery in the developed world (Kubba, Pearson
and Birchall 2000), and over 30,000 VTs were inserted in England during 2005/6 (HES 2010). OME and related acute middle ear infections (acute otitis media, AOM) are the leading cause of primary care visits (Freid, Mukuc and Rooks 1998), and the most frequent reason for antibiotics or surgery (Keyhani, et al. 2008) (Rovers, et al. 2004). Annual costs in the USA were estimated to be in excess of $5 billion in 1998, but this is likely to have risen by now, and indirect costs are likely to be higher still (Bondy, et al. 2000).

OME, acute otitis media and recurrent AOM (RAOM, 3 or more episodes in 6 months) are closely related clinical conditions (Alho, et al. 1995), with AOM representing an acute infective (bacterial and/or viral) process, whereas OME is characterised by the presence of a middle ear effusion in the absence of symptoms and signs of acute inflammation (Kubba, Pearson and Birchall 2000). AOM differs from OME in that AOM is characterised by ear pain and fever in a child that is systemically unwell. In some cases the pain is followed by ear discharge as the tympanic membrane perforates, and the resultant release of pus under pressure leads to improvement in pain. Several other clinical entities are characterised by inflammation of the middle ear ("otitis media"), shown in Fig 2.1.1, with ear anatomy illustrated in Fig 2.1.2.
Otitis Media

acute

acute otitis media: acute infection in middle ear
recurrent acute otitis media: 3 or more episodes in 6 months

chronic

otitis media with effusion: middle ear effusion in absence of symptoms or signs of acute inflammation
chronic suppurative otitis media: middle ear inflammation, usually with tympanic membrane perforation
cholesteatoma: squamous epithelium in the middle ear, leading to inflammation

Fig 2.1.1: Different types of Otitis Media (top box) and clinical photographs showing normal TM (A), acute otitis media (B), dull tympanic membrane in otitis media with effusion (C), ear drum perforation (D), retraction (E) and cholesteatoma (F). ME: middle ear. TM: tympanic membrane. A and D-F (Flint, et al. 2010); B and C (Datta 2012).
Fig 2.1.2: Ear anatomy (NYSDOH 2011)
OME is diagnosed (Browning 1998) on the basis of a history of hearing loss, speech delay (typically mispronunciation rather than lack of vocabulary), or educational concerns, in some children associated with episodes of AOM (ear pain, fever, perhaps followed by discharge). Otoscopy can reliably make the diagnosis if carried out by an experienced operator, with diagnosis being confirmed by tympanometry and hearing assessment. Tympanometry measures the sound reflected from the ear drum while the pressure of the external canal is varied by the operator. Normally the maximum sound reflection occurs at atmospheric pressure, but in OME the sound is typically all absorbed by the fluid in the middle ear leading to a flat tympanogram (Fig 2.1.2). A flat tympanogram is also obtained in patients with tympanic membrane perforation, but this is easily distinguished from OME by clinical examination and by the finding of a large canal volume measured at tympanometry, as the volume measured then represents not only the volume of the ear canal but also the volume of the middle ear measured through the perforation. On audiometry, whether standard pure tone audiometry or methods suitable for children, a mild conductive hearing loss is present (Fig 2.1.3); hearing level is by convention measured on a biological logarithmic scale compared to normal hearing (decibel Normal Hearing Level, dB NHL), with anything between 0 and 20 dB NHL considered within normal limits.
Fig 2.1.2: Tympanometry. A: normal peaked tympanogram. B: flat tympanogram typical of OME (Drtbalu 2010).

Fig 2.1.3: Audiometry showing normal hearing in the right ear (A) and mild conductive loss on the left (B) (McGill 2010).
2.2 CURRENT MANAGEMENT OF OME

2.2.1 Ventilation Tubes and NICE Guidelines

Ventilation Tube (Fig 2.2.1.1) insertion is currently the only effective and UK-recommended treatment to restore hearing (NICE 2008) but requires a general anaesthetic in children. A variety of different tubes are available, ranging from the short term (typically 6-9 months) Shepard tubes, through Shah and Reuter bobbins, to T-tubes and Permavents that stay in place for several years. The longer a tube stay in the more likely it is to lead to problems, with a persistent perforation rate of 5.6% reported in Shah grommets (Yaman, et al. 2010), but occurring in more than 10% if long term ventilation tubes are inserted (Saito, et al. 1996).

Fig 2.2.2.1: Ventilation tubes

Numerous medical treatments have been tried in order to avoid surgery. Antibiotics particularly have received considerable attention (Rosenfeldt and Bluestone 1999) (Rovers, et al. 2004). Although oral antibiotics are effective in the short term, increasing OME resolution rate by about 15% (Rosenfeldt and
Bluestone 1999) (Rosenfeld and Post 1992), this benefit is short lived and there is no significant advantage after a few month (Gunasekera, et al. 2009) (Rosenfeldt and Bluestone 1999) (Rovers, et al. 2004) (Williams, et al. 1993). Antibiotics are not a recommended treatment in the UK (NICE 2008), nor are steroids, decongestants, or antihistamines.

The prevailing current treatment strategy, based on NICE guidelines on the surgical management of OME (NICE 2008), is VT insertion in children with symptomatic persistent bilateral OME documented over a period of 3 months with a hearing level in the better ear of 25–30 dB NHL or worse. The observation period is suggested due to high incidence of spontaneous resolution, and only children that are being affected by OME (for example with speech delay or schooling problems) should undergo surgery. The guidelines are clear that surgery is inappropriate for children that have OME only for a short time period or in cases where OME has little effect on child’s function and development; this contrasts with older clinical practice and trials that assessed children that were very much at the mild end of the spectrum.

The aim of any guideline (systematic statement of best evidence-based practice to assist doctors and patients in decision making about appropriate healthcare in specific circumstances (NHS Centre for Reviews and Dissemination 1994)) such as NICE Guidance on surgical management of OME is to inform and standardise best practice. However, whilst guidelines should lead to improved patient care, this is by no means seen universally (Freemantle, et al. 2000) (NHS Centre for Reviews and Dissemination 1994)

The odds of surgery for OME/AOM were noted to vary 4 fold across different regions of Norway in 2002 (Karevold, et al. 2007), and up to 10 fold for AOM across different countries (UK lowest) (Schilder, Lok and Rovers 2004). There was also a 10 fold variation in VT rates within Ontario in Canada in the late 1990s (Coyte, et al. 2001), this variation being larger than that seen in almost any other procedure. Higher socio-economic status and the enthusiasm of the primary care doctor for VTs were associated with increased VT rates. An American study in 2002 found that >90% of VTs were not inserted in accordance with guidelines in place at the time, and 80% were not appropriate according to criteria developed by an expert panel for the study (Keyhani, et al. 2008). Even after excluding children with other conditions predisposing to a poor developmental outcome, those requiring other surgery, or those that have had VTs before, the number of appropriate VTs was still less than 40%.

Going back several decades, the number of VTs in the UK was considered excessive, and guidelines published in 1992 (NHS Centre for Reviews and Dissemination 1992) emphasised the fact that for many children OME was a temporary phenomenon due to spontaneous resolution and watchful waiting should be the norm for most. The rates of OME surgery in England halved during the 1990s, and although the guidelines undoubtedly contributed, there was already an existing downwards trend in surgery rates (Black and Hutchings 2002).
Several possible explanations exist for the variation in surgery rates, ranging from differences in clinical opinion on diagnosis and effectiveness of treatment, to parental preferences, and perhaps even financial factors. Of course the problem may not be that surgeons are ignoring the guidelines, it may be that the guidelines are over-restrictive and do not allow personalisation of care to each individual child.

Whilst the recent NICE guidelines on the surgical management of OME have clarified best practice, it is not yet known whether they have had an impact on OME treatment, nor whether there is a variation in compliance across different hospitals.

2.2.2 Adenoidectomy

Despite VT insertion, about a quarter of children will require further surgical treatment within 2 years (Gates, Avery and Prihoda 1987). In order to reduce recurrence, adenoidectomy as an adjunct to VT insertion has been investigated by several studies (Rosenfeldt and Bluestone 1999).

Gates et al (Gates, Avery and Prihoda 1987) followed up 254 children aged 4-8 years for 2 years. VTs+ad reduced time with effusion (26% of visits vs. 35%) and halved the need for further surgery (14% vs. 28%). Maw (Maw and Bawden 1994) followed up 222 children aged 3-9 years for up to 10 years. At 5 years, VTs+ad had half the re-treatment rate of VTs alone: 34 vs. 68%. At 10 years mean numbers of VTs needed were 1.5 and 2.5, respectively; +ad also appeared to benefit hearing, the probable chief basis for the reduced
further treatment, for up to 4 years. A trial comparing VTs+ad with myringotomy +ad in 3-7 year old children concluded that hearing in both groups improved, but using VTs instead of a myringotomy conferred no additional advantage, the implication being that it is the adenoidectomy that gets children better (Popova, Varbanova and Popov 2011). A RCT looking at adjuvant adenoidectomy in 4-9 year-old children found that while VTs were responsible for most of the initial benefit (better than myringotomy), adjuvant adenoidectomy increases the likelihood of restoration of normal middle ear function at least up to 2 years after surgery (Black, et al. 1990). In contrast, a study in children aged 2-3 years found that adenoidectomy conferred no advantage in terms of time with effusion over VTs alone (Casselbrant, et al. 2009), and another (small – 72 patients) trial found that neither VTs nor VTs+ad influenced OME resolution compared with no surgery at 12 months (Dempster, Browning and Gatehouse 1993). Importantly, a large study of >50,000 children aged under 10 years found that adenoidectomy in addition to VTs reduced the need for subsequent surgery from 36% to 20% (Kadhim, et al. 2007).

A 1992 review in Effective Health Care concluded that adding adenoidectomy to VTs only had a small effect up to 12 months post op (NHS Centre for Reviews and Dissemination 1992). A Cochrane review in 2010 concluded that adenoidectomy has a significantly beneficial effect on OME resolution, although only a small benefit to hearing (van den Aardweg, et al. 2010).
In the UK adjuvant adenoidectomy is not recommended in the absence of persistent and/or frequent upper respiratory tract symptoms (NICE 2008). Whilst American guidelines agree with UK stance in the case of primary surgery, they recommend that adenoidectomy is undertaken in conjunction with any revision surgery (Rosenfeld, et al. 2004). This is because the role of adjuvant adenoidectomy is set in part by issues of risk (chiefly bleeding) rather than purely (cost-) effectiveness. There is not an evidence-based consensus about exactly who should receive adenoidectomy or when. The fact that many children do not require a second set of tubes is used to argue against adenoidectomy. There is a great variation in the number of children receiving adenoidectomy in different countries, but on the whole the number of children having adenoidectomy appears to be reducing (Haapkyla, et al. 2008). The NICE guidelines on surgical management of OME commented on the generally poor quality of adenoidectomy trials, calling for better quality research to evaluate any benefits of adenoidectomy (NICE 2008).

A great degree of controversy surrounds adjuvant adenoidectomy in conjunction with VTs to try and reduce high rate of further surgery after VT extrusion, leading to the setting up of a multicentre UK study on glue ear treatment. The MRC Trial of Alternative Regimens on Glue ear Treatment (TARGET) examined children up to 2 years after randomisation to VTs, VTs+ad or watchful waiting, and found that adjuvant adenoidectomy benefits hearing for the 2 year duration of the trial (Browning 2012) (MRC Multicentre Otitis Media Study Group 2001a) (MRC Multi-Centre Otitis Media Study Group 2001b). However, the follow-up period was only 2 years, but OME often lasts
much longer. Therefore, longer-term data is also of value, warranting supplementary studies beyond the TARGET 2 years (TARGET and the long-term supplementary study presented in this thesis is described in more detail later).

2.2.3 Evaluating OME treatment: which children to study and for how long?
OME is a temporary phenomenon in most children. When discovered on screening of asymptomatic children, it resolves in 63% by three months and in 88% by one year (Rosenfeldt and Bluestone 1999). The NICE guidelines have taken this into account as treatment is inappropriate for most children with incidentally discovered, short-term, unilateral or asymptomatic OME.

The group of children where surgery may be considered are those with bilateral OME persisting at least three months. This group has a much lower spontaneous resolution rate (Maw and Bawden 1994) (Rosenfeldt and Bluestone 1999) (Table 2.2.3.1), with the median duration of OME being 7.8 years (Maw and Bawden 1993). The surgical candidates, therefore, are a select group of children in whom untreated OME often follows a protracted relapsing and remitting course.
Table 2.2.3.1: Spontaneous resolution rate in children with bilateral OME that has already persisted for 3 months at the start of the trial (Maw and Bawden 1994) (Rosenfeldt and Bluestone 1999).

This complex evolution of the natural history of OME raises difficulties for the evaluation of treatments, as some patients may improve without treatment, whereas others suffer recurrence after VT extrusion. Any RCT therefore faces the issue of appropriate time scale through which to evaluate a treatment.

The natural efficacy of most VTs is in the region of 6-9 months, the time that VTs stay in place before being spontaneously ejected. Therefore, evaluating VTs over 12 months is a sensible initial trial duration that takes in the VT stay period. However there is no such clearly defined evaluation period for treatments other than VTs, for example adjuvant adenoidectomy or any new
techniques. Most trials evaluate outcomes over at least 2 years to take account not only of VT stay time but also of the recurrence after VT extrusion, but longer term data is rare (Rosenfeldt and Bluestone 1999).

The two year follow up was also adopted by TARGET, the Trial of Alternative Regimens in Glue Ear Treatment (Browning 2012) (MRC Multi-Centre Otitis Media Study Group 2001b) (MRC Multicentre Otitis Media Study Group 2001a), a major UK multi-centre RCT designed to evaluate OME treatment over a 2 year period on children aged 3.5 to 7 years. TARGET’s main entry criterion was 20 dB NHL or worse in the better-hearing ear on initial visit, and the same again after 3 months. Those qualifying and accepting randomisation (376 children) were then randomised to VTs, VTs+ad, or further active observation (FAO). A further adjunct cohort of 56 children, with hearing worse than 40dB NHL that were thought to have an overriding need for treatment, were also treated and given discretionary adenoidectomy. Subject to reservations about non-randomised analysis, this group, known as “obliged to treat” (OTT), boosts the numbers for comparing VTs+ad with VTs alone or FAO.

Children were followed up for 2 yrs. There was spontaneous remission in about a third of FAO children; however, overall 62% of children allocated to FAO switched to a surgical treatment arm within 1 year, emphasising the clinical need of this OME population as a whole. VTs led to better hearing outcomes initially, but after 1 year there was no advantage of VTs over FAO.
TARGET entry criteria ensured that only the severe spectrum of OME was captured, as evidenced by the high switching from FAO to surgery. Studies that adopt less stringent entry criteria have questioned the benefits of VTs. For example, the widely quoted Paradise study (Paradise, et al. 2007) included children purely on the basis of the presence of effusion even if unilateral, accepted 15dBHL as the audiometric criterion, and analysed largely children under 2 years old. Their findings of limited benefits of VTs are not surprising as they studied a group of children that likely did not have much of a problem in the first place. A benefit of VTs in terms of language, writing and behaviour, both while VTs are in-situ and after extrusion, has been demonstrated by UK-based studies on the more severely affected children (Hall, Maw and Steer 2009) (Wilks, et al. 2000).

Although most OME trials look at a two year period that encompasses VT stay time and the period immediately after extrusion, the chronic nature of OME should ideally also be taken into account, although exactly how long any trial should last is unclear.
2.3 AETIOLOGY OF OTITIS MEDIA WITH EFFUSION

Despite its major socio-economic and health care importance, the aetiology of OME is only now being elucidated (Rovers, et al. 2004). It is a chronic inflammatory condition affecting the middle ear mucosa resulting in mucus secretion that accumulates in the middle ear cleft (Kubba, Pearson and Birchall 2000). The middle ear mucosa constantly secretes mucus, which is then moved down the Eustachian tube. The mucus has a protective function, as it provides a physical barrier and prevents adhesion of bacteria to mucosa. Upon irritation, the mucosa responds by increasing the production of mucus and secretion of altered mucus types (Kubba, Pearson and Birchall 2000), goblet cells proliferate, and new mucous glands form (Bak-Pedersen and Tos 1976) (Tos 1980) (Tos and Caye-Thomasen 2002).

In addition to mucins, middle ear effusions also contain water, cells, cell debris, electrolytes and various other high molecular weight compounds such as proteins, lipids, and DNA. Bacteria and their product have also been identified, as have immunoglobulins, lysozyme, lactoferrin, complement, leukotrienes and cytokines. While many of these may be present as a result of bacterial inflammation, mucins are actively secreted into the effusion (Smirnova, et al. 2003); OME is an exudate, not a transudate.

Whilst it is clear that OME develops as a result of mucosal irritation, the cause of the initial inflammatory stimuli has been difficult to establish (Kubba, Pearson and Birchall 2000).
2.3.1 Bacteria in OME

Whilst OME is a chronic inflammatory condition on histology, the cause of the inflammatory stimulus that leads to production of mucins and an effusion has been difficult to identify. Some of the risk factors for OME (young children, association with AOM, day-care, many siblings, lack of breastfeeding) suggest an infective aetiology, but OME is not characterised by symptoms and signs of acute inflammation that would be expected in a typical acute bacterial infection with a bulging red tympanic membrane, pain and fever.

The role of bacteria in OME has therefore been controversial. In most studies bacteria could be cultured in fewer than half of samples, ranging from 21 to 70% (Bluestone, Stephenson and Martin 1992) (Gok, et al. 2001) (Hall-Stoodley, Hu, et al. 2006) (Hendolin, et al. 1997) (Matar, et al. 1998) (Poetker, et al. 2005) (Schousboe, et al. 2001). Although this may suggest that bacteria are not important in OME, it contrasts with studies examining effusions for the presence of bacterial nucleic acids by polymerase chain reaction (PCR), which have demonstrated bacterial DNA typically in excess of 80% of effusions (Hendolin, et al. 1997) (Kubba, Pearson and Birchall 2000) (Post, Preston, et al. 1995) (Rayner, et al. 1998). However the presence of bacterial nucleic acids does not equate to the presence of viable bacteria, and components of effusion samples have been shown to inhibit nuclease activity, perhaps leading to persistence of RNA and DNA even if bacteria are no longer viable (Peizhong, et al. 2000).
A possible explanation for the discrepancy between high PCR-positive rate and low culture-positive rate in OME is involvement of biofilms (Fergie, et al. 2004), as these may give culture-negative results in the laboratory (biofilms are described later). Indeed, biofilms have been identified on human middle ear mucosa in children with OME and/or recurrent AOM in 65 - 92% of cases, but not in any control samples studied (Thornton, et al. 2011)(Hall-Stoodley et al 2006). Biofilms have also been identified in animal models of OME (Ehrlich, Veeh, et al. 2002) (Post 2001). However, a study of middle ear mucosal biopsy at time of cochlear implant showed biofilms in 2 out of 45 cases without evidence of previous otologic problems or abnormalities, suggesting that biofilms may exist in the asymptomatic middle ear (Tonnaer, Mylanus, et al. 2009). Biofilms have also been found in patients with cholesteatoma (Chole and Faddis 2002) and chronic suppurative otitis media (Homøe, et al. 2009).

In addition to biofilms, bacterial persistence may also occur due to intracellular infection (Coates, et al. 2008), perhaps co-existing with biofilm (Thornton, et al. 2011).

A wide range of bacteria have been implicated in OME. The commonest bacterial isolates are *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*, with *Staphylococcus aureus* and many others also frequently reported (Bluestone, Stephenson and Martin 1992) (Hyden, Akerlind and Peebo 2006).

In addition to suggestions that OME is a biofilm disease, it is also thought that the benefits of adenoidectomy are linked to removal of nasopharynx biofilms.
Adenoid tissue acts as a reservoir of bacteria: bacteria in the middle ear get there by ascending via the Eustachian tube from the nasopharynx and adenoid tissue (Tonnaer, Rijkers, et al. 2005). Adenoidectomy alters the nasopharyngeal bacteria, reducing the incidence of carriage of *Haemophilus influenzae* and *Streptococcus pneumoniae* (Aarts, et al. 2010), and adenoid tissue of children with OME has been shown to contain bacterial biofilms (Hoa, et al. 2010) (Kania, et al. 2008) (Nistico, et al. 2011) (Winther, et al. 2009). However, adenoid tissue contains bacteria even in absence of OME (Nistico, et al. 2011), and the adenoid bacteria may be no different in those children with OME from those without (Almac, et al. 2009).

**2.3.2 Other theories of OME aetiology**

Several other theories of OME aetiology have been investigated in the past (Kubba, Pearson and Birchall 2000). The unifying pathway is inflammation leading to production of more and thicker mucus which accumulates in the middle ear, in conjunction with impaired mucociliary clearance due to the increased mucus viscosity. Figure 2.3.2.1 presents an integrated model of OME aetiopathogenesis.
Eustachian tube function has received a lot of attention, but aetiology of OME is much more complex than Eustachian obstruction. If OME developed as a result of a physical Eustachian tube obstruction, then the fluid would be a transudate and not an exudate (Kubba, Pearson and Birchall 2000). Another factor pointing against Eustachian tube blockage can be inferred from results of treatment with VT insertion: one study of children having VTs inserted aspirated fluid only on one side but not the other. If the Eustachian tube was blocked, then the fluid left in the middle ear should drain out through the VT, but in fact the effusion dissipates down the Eustachian tube, suggesting that there was no physical blockage of the Eustachian tube (Sade, Halevy and Hadas 1976). Eustachian tube dysfunction (as opposed to obstruction) may
be involved as the tube is the likely source of bacteria in the middle ear ascending from the adenoids / nasopharynx, and its function may be affected by acute bacterial or viral infections (Rosenfeldt and Bluestone 1999).

Smoking causes impaired ciliary action, but is in itself unlikely to be the predominant cause of OME as children not exposed to passive smoking still get OME (Sophia, et al. 2010). Ciliary beat frequency in children with effusion is no different from those without (~11Hz) (Wake and Smallman 1992). Viral DNA has also been identified in the middle ear, but a causal relationship to OME has not been established (Pitkaranta, et al. 1998). Atopy is a weak risk factor at the most (Caffarelli, et al. 1998) (Sophia, et al. 2010). Diesel exhaust fumes have been shown to decrease cell viability, induce inflammation, and increase mucin expression in middle ear epithelial cell cultures (Song, et al. 2012).

Genetic aetiology has been investigated in mouse models (Hardisty-Hughes, et al. 2006) (Parkinson, et al. 2006). However, there is no good animal model of OME, and at least some of the animal mutants with OME have associated craniofacial abnormalities. Also, the effusion composition has not been studied to demonstrate comparability with humans, and the observation of different effusion components between the right and the left ear (Johnson, et al. 1997) suggests that genetic influences alone are unlikely to be responsible for OME, although they may influence the host interaction with pathogens or the inflammatory response.
The aetiology of OME is unclear, but the role of bacteria and biofilms warrants further investigation using new methods that can demonstrate bacteria in the middle ear effusion.
2.4 BACTERIAL BIOFILMS AND THEIR TREATMENT

2.4.1 Pathogenesis of biofilm infections
Bacteria exists in two forms, planktonic and biofilms. Planktonic bacteria are free-floating unicellular organisms, allow rapid dissemination over a large area, and have been successfully targeted by antibiotics. On the other hand, bacterial biofilms, now known to be present in virtually all natural and pathological environments, are emerging as major causes of human disease (Vlastarakos, et al. 2007), being focused on persistence and characterised by their recalcitrance to antibiotics and clearance by host defence mechanisms (Donlan and Costerton 2002). It has been estimated that >60% of infections are caused by biofilms (Lewis, Riddle of Biofilm Resistance 2001). Although probably first described in the seventeenth century by Anton van Leeuwenhoek’s examination of his own dental plaque, universal acceptance of biofilms in human pathogenesis has only occurred in the last few decades (Schinabeck and Ghannoum 2006). Bacteria grown as colonies on an agar plate are thought to be most similar to planktonic bacteria (Mikkelsen, et al. 2007), so the study of biofilms requires new, different methods.

Biofilms are structured communities of bacteria embedded in a self-produced extracellular matrix attached to a surface or interface (which may be surrounding mucus or fluid) (Yang, et al. 2008), and exhibiting altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton 2002) (Hall-Stoodley and Stoodley 2009) (Schinabeck and Ghannoum 2006). The last characteristic distinguishes them from those
bacteria attached to surfaces but not exhibiting the altered phenotype, for example bacteria growing on agar. Biofilms can be composed of single or multiple species. They are not homogeneous monolayers of bacteria on a surface, but are composed of bacterial micro-colonies (accounting for ~15% of the biofilm volume) encased in matrix (~85%) (Fig 2.4.1.1) (Donlan and Costerton 2002) (Lawrence, et al. 1991) (Rasmussen and Lewandowski 1998).

When a planktonic bacterium arrives at a surface it become reversibly attached in the first instance (Pascual 2002) (Schinabeck and Ghannoum 2006). The close proximity allows irreversible adhesion to occur, which

Figure 2.4.1.1: Schematic biofilm structure
depends on direct interaction between bacterial ligands and the host / surface (Gristina 1987). Adhesion of bacteria to a surface triggers a change in phenotype (Otto 2008), with biofilms forming as fast as within 24 hours (Nickel, et al. 1985) (Oliveira, et al. 2007) (Vorachit, et al. 1993). Once irreversibly adherent, cell proliferation and biofilm formation occurs (Fig 2.4.1.2) (Schinabeck and Ghannoum 2006). In addition to tissue surfaces, biofilms have also been identified attached to collagen gel matrix (Werthen, et al. 2010) and mucus (Corazziari 2009) (Werthen, et al. 2010) (Winther, et al. 2009) (Yang, et al. 2008). In OME, biofilms could be attached to mucus as well as mucosa, and the resultant inflammatory stimulus may then lead to a middle ear effusion (Hunter, et al. 1999) (Schousboe, et al. 2001) (Smirnova, et al. 2002) (Sun, et al. 2012).

Figure 2.4.1.2: Biofilm life cycle
Biofilms cause disease in a number of ways (Donlan and Costerton 2002). Parts of the biofilm can break off (Gristina 1987) (Stoodley, et al. 2001) when the tensile strength is exceeded, resulting in septic embolisation, and biofilms can also shed planktonic bacteria leading to distant spread. Bacteria can induce a host response through the production of endotoxins (Vincent, Tibi and Darbord 1989), but biofilms themselves may be less susceptible to host immune response (Hall-Stoodley and Stoodley 2009) (Shiau and Wu 1998) (Ward, et al. 1992) (Yasuda, et al. 1994), and the close proximity of bacteria may encourage the transfer of plasmids even between bacteria of different genera (Ehlers and Bouwer 1999).

2.4.2 Criteria for a biofilm infection

Many infections have been associated with biofilm formation (Donlan and Costerton 2002) (Hall-Stoodley and Stoodley 2009). Parsek and Singh suggested that the criteria in Table 2.4.2.1 should be fulfilled for a disease to be considered a biofilm infection (Parsek and Singh 2003).

<table>
<thead>
<tr>
<th>Parsek and Singh criteria for biofilm infections</th>
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<tbody>
<tr>
<td>Bacteria are adherent to substratum or a surface</td>
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<tr>
<td>Bacteria living in cell clusters or micro-colonies encased in extracellular matrix</td>
</tr>
<tr>
<td>Infection generally confined to a particular location</td>
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<tr>
<td>Infection difficult or impossible to eradicate with antibiotics, despite the responsible bacteria being susceptible when in the planktonic state</td>
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Table 2.4.2.1: Parsek and Singh criteria for biofilm infections (Parsek and Singh 2003)
2.4.3 Studying bacterial biofilms \textit{in vitro}

A number of different ways of studying biofilms \textit{in vitro} have been described, with several important parameters to consider (Donlan and Costerton 2002):

- Medium: composition, temperature, presence of antimicrobials
- Inoculum: identity of organism, number of cells
- Hydrodynamics: flow rate, presence of shear
- Substratum: roughness, chemistry, conditioning film

The \textit{in vitro} biofilm models are broadly similar in that they all rely on the provision of a surface for bacterial attachment and a nutrient environment. Some surfaces are more conducive to biofilm formation than others, with latex and silicone being most supportive of bacterial attachment, followed by Poly(vinyl chloride), Teflon (poly(tetra fluoro ethylene)), polyurethane, stainless steel and titanium (Darouiche 2001) (Pascual 2002) (Schinabeck and Ghannoum 2006), although not all studies agree (Bayston, Ashraf and Barker-Davies, et al. 2007). Surfaces that are rough or irregular have enhanced bacterial attachment, as do those that are hydrophobic or positively charged as they attract bacteria that are predominantly hydrophobic and negatively charged in their planktonic state (Darouiche 2001) (Schinabeck and Ghannoum 2006). However, the characteristics of any implanted material can be altered by the presence of a “conditioning film” composed of several host components (fibronectin, fibrinogen, fibrin, albumin, collagen, elastin, von Willebrand factor), which may in some circumstances increase bacterial adherence (Pascual 2002) (Schinabeck and Ghannoum 2006). Rather counter
intuitively, bacterial adhesion is promoted by shear forces, with biofilms formed in high shear environments being stronger (Donlan and Costerton 2002).

In order to study OME biofilms and their eradication in the laboratory setting, a new *in vitro* model is required. Demonstration of biofilm structure as well as its functional characteristics, reduced susceptibility to antibiotics, will have to be shown. Because high dose antibiotics delivered locally over several weeks are likely to be required for biofilm eradication, the new model must be able to incorporate the addition of antibiotics or a modified release device sized for use in the middle ear. Although a number of well known biofilm systems have been used by other researchers, including the commercially available Calgary Biofilm Device (Minimum Biofilm Eradication Concentration (MBEC) assay system) (Ceri, Olson and Stremick, et al. 1999) and the CDC biofilm reactor (Goeres, et al. 2005), as well as the modified Robbins device (Lewis, Riddle of Biofilm Resistance 2001), the dimensions of these are not suitable for examining the effects of an added modified release device intended for use in the middle ear. Therefore a new *in vitro* biofilm model will have to be developed.

In order to measure bacteria in biofilms on a surface, the biofilm can be removed from the surface using sonication or enzymes, and the resulting bacterial suspension plated to count the number of colony forming units (Ceri, Olson and Stremick, et al. 1999). Other methods assess biofilms *in-situ* by measuring the turbidity of nutrient broth (Ceri, Olson and Stremick, et al. 1999)
or the intensity of staining with crystal violet (Djordjevic, Wiedmann and McLandsborough 2002), but this does not allow the counting of colony forming units. An important step in the study of biofilms is the use of resuscitation tests where biofilms previously exposed to a treatment agent are then placed into fresh nutrient broth to determine whether any bacteria have persisted, and are capable of re-growing once the inhibitory effect of antibiotics is withdrawn.

2.4.4 Treatment of biofilm infections

Biofilms show inherently low susceptibility to treatment with antibiotics, typically requiring from 10 to 1000 times higher antibiotic levels than those required to inhibit planktonic bacteria (Ceri, Olson and Stremick, et al. 1999) (Donlan and Costerton 2002) (Nickel, et al. 1985) (Olson, et al. 2002) (Vorachit, et al. 1993). This reduced susceptibility to antibiotics, termed recalcitrance, is distinct from antibiotic resistance occurring as a result of genetic alterations. Resistance (Lewis 2001) is the ability of an organism to grow in the presence of elevated levels of an antimicrobial, yet biofilms do not show this as the bacteria in their planktonic state are as sensitive to antibacterials as expected; only when grown in a biofilm is the reduced susceptibility, the recalcitrance, evident. This recalcitrance arises as a result of several possible factors (Hall-Stoodley and Stoodley 2009) (Lewis, Riddle of Biofilm Resistance 2001) (Sihorkar and Vyas 2001).

- Bacteria in biofilms are slow growing, rendering them relatively less susceptible to antibiotics (DuGuid, et al. 1990) (Evans, et al. 1990).
- Adhesion to a surface and change to the biofilm phenotype influences the expression of a number of genes and physiologic parameters that may
affect susceptibility to antibiotics (Dagostino, Goodman and Marshall 1994).

• Within the biofilm structure a gradient of oxygen, pH, or nutrient levels exists (Rasmussen and Lewandowski 1998), and this gradient may affect antibiotics directly or change the growth of bacteria in the anaerobic micro-niche at the centre of the biofilm (Hall-Stoodley and Stoodley 2009) (Proctor and von Humboldt 1998) (Sihorkar and Vyas 2001).

• Biofilms contain “persister cells” that are not affected by antibiotics even when other biofilm bacteria are killed, perhaps due to expression of specific genes capable of shutting down antibiotic targets to create a dormant tolerant cell (Lewis 2001). This temporary state is distinct from the permanent and heritable resistance characterised by prevention of antibiotic binding to target in a cell that continues to be metabolically active. It is also not due to bacteria being at a specific point in the cell cycle and, although related to growth phase, is not identical with simply non-growing cells (Lewis, Spoering, et al. 2006) (Shapiro, Nguyen and Chamberlain 2011).

• Antibiotics have to penetrate the biofilm matrix to get to the bacteria, but the matrix may affect the diffusion of antibiotics so that their effectiveness is reduced. Not all antibiotics are thought to be affected equally, with rifampicin, clindamycin and macrolides thought to be relatively uninhibited (Souli and Giamarellou 1998).

• These recalcitrance factors can act singly or in combination (Donlan and Costerton 2002).
As biofilms do not behave the same as planktonic bacteria, the use of planktonic bacteria-based Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) when discussing effects of antibiotics is inappropriate, leading to the use of Minimum Biofilm Eradicating Concentration (MBEC) instead (Ceri, Olson and Morck, et al. 2006). MBEC is typically in the region of 10 to 1000 times higher than MIC (Ceri, Olson and Stremick, et al. 1999) (Donlan and Costerton 2002) (Nickel, et al. 1985) (Olson, et al. 2002) (Vorachit, et al. 1993), and treatment over several weeks is typically required (R. Donlan 2008). However, whether that high antibiotic level is required for the full duration of treatment is not known, and it may be that an initial high level followed by a lower antibiotic level is satisfactory.

Several broad approaches may be used against biofilm infection (Aslam 2008) (Donlan and Costerton 2002) (Sihorkar and Vyas 2001):

- Prevention of initial contamination, e.g. by adhering to strict aseptic techniques.
- Prevention of initial microbial attachment and biofilm development e.g. with antibiotic impregnated devices (Bayston, Ashraf and Bhundia 2004) (Bayston, Fisher and Weber 2009) or other device modification (Biedlingmaier, Samaranayaki and Whelan 1998) (Furno, et al. 2004).
- Eradication of established biofilms with antibacterial agents (R. Donlan 2008) (Sihorkar and Vyas 2001), delivered either systemically or locally: antibiotic bone cement, catheter antibiotic-lock therapy (R. Donlan 2008) (Fernandez-Hidalgo, et al. 2010), or novel drug delivery systems such as
liposomes or biodegradable polymers (Sihorkar and Vyas 2001) (Smith 2005). Additionally, novel techniques may be used such as ultrasound (Ensing, et al. 2006) (Huang, et al. 1996), low-strength electrical fields (Blenkinsopp, Khoury and Costerton 1992), degradation of extracellular matrix (Johansen, Falholt and Gram 1997), inhibition of quorum sensing (Bassler and Losick 2006) (Diggle, Crusz and Camara 2007) (P. Williams 2007), disruption of biofilm-related genes, or indeed a combination of the above in a smart system that detects and treats colonising infection (Ehrlich, Stoodley, et al. 2005).

- Removal of infected surface.

Eradication of established biofilm infections is notoriously difficult (Donlan and Costerton 2002) (Lewis 2001), but it is likely that high antibiotic concentrations with extended exposure periods will be required, with certain antibiotics (e.g. rifampicin and clindamycin) appearing to be better against biofilms than others (Lee, et al. 2006) (Souli and Giamarellou 1998). Those two antibiotics were therefore chosen for the study of biofilm eradication in this thesis. Additionally, the combination of two antibiotics minimises emergence of resistance (D'Agata, et al. 2008).

2.4.5 Rifampicin and clindamycin

The interaction between antibiotics and planktonic bacteria is described by MIC and MBC, and that between antibiotics and biofilms by the MBEC as explained above. Although these describe in vitro activity of antibiotics, they do not describe the time course. Antibiotics are divided into two broad
categories, time-dependent and concentration-dependent (Finch and Gander 2006). Concentration-dependent antibiotics (aminoglycosides, quinolones) show an increase in the rate and the extent of bactericidal activity with increased concentration. With time-dependent antibiotics (beta-lactams, linezolid, tetracyclines) the rate and extent of killing is dependent of time above a minimum concentration, but further increases in antibiotic concentration do not increase the cidal activity. Some time-dependent antibiotics also exhibit post-antibiotic effects. Antibiotics that are bacteriostatic inhibit the growth and reproduction of bacteria without killing them (relying on the immune system to kill bacteria), whereas bactericidal antibiotics kill bacteria.

For the study of biofilm eradication, rifampicin and clindamycin were chosen. They appear to be better than other antibiotics at eradicating biofilms (Lee, et al. 2006) (Souli and Giamarellou 1998), the combination of two antibiotics minimises emergence of resistance (D'Agata, et al. 2008), and between them they cover common OME pathogens.

In terms of their pharmacodynamic activity, the stratification of rifampicin and clindamycin into different types of antibiotics is not clear-cut. Rifampicin can be bacteriostatic or bactericidal depending on dose and exposure time, and exhibits time-dependent and also concentration-dependent killing (Bakker-Woudenberg, et al. 2005) (Gumbo, et al. 2007). Similarly, clindamycin can be bacteriostatic or bactericidal depending on dose and exposure time, and
exhibits time-dependent (Klepser, et al. 1996) and also concentration-dependent killing (Aldridge and Stratton 1991).

Clindamycin, C_{18}H_{33}ClN_{2}O_{5}S (Fig 2.4.5.1), molecular weight 425.0, is a lincosamide antibiotic that binds to the 50s subunit of the ribosome and interferes with protein synthesis. It is active against aerobic Gram-positive cocci including staphylococci and streptococci, and against anaerobic Gram-negative rods, but most aerobic Gram-negative bacteria (Haemophilus, Moraxella) are resistant.

![Clindamycin structure](Fig 2.4.5.1: Clindamycin (Sigma 2012))

Rifampicin, C_{43}H_{58}N_{4}O_{12} (Fig 2.4.5.2), molecular weight 823.0, inhibits DNA-dependent RNA polymerase. It is active against most Gram positive cocci including Streptococci and Staphylococci, Haemophilus influenzae, as well as mycobacteria (Thornsberry, Hill, et al. 1983) (Thornsberry, Ogilvie, et al. 1999).
Although a single step mutation renders bacteria resistant and rifampicin is therefore not recommended for monotherapy, it has been found to be particularly effective in biofilm infections. Much information exists on using rifampicin in combination with other antibiotics, showing impressive results in the management of biofilm infections with a strong synergistic bactericidal activity (Barriere 2006).

Thus eradication of biofilms with antibiotics appears to be a promising strategy, with rifampicin and clindamycin likely to be a good choice. Certainly treatment of OME could be improved, and the current high need for repeat VT insertion is not surprising if OME is a biofilm infection, because whilst VTs may remove the effusion to improve hearing, they do not deal with the underlying problem of a biofilm infection. A new, better treatment strategy based on understanding of OME pathogenesis is thus required. Biofilm eradication requires high antibiotic levels over several weeks, and therefore the use of a modified release formulation to deliver high dose antibiotics direct
to the middle ear is a potentially useful future strategy. Local delivery achieves high enough antibiotic levels, and the use of a modified-release formulation ensures treatment over a sufficiently long period. An understanding of modified-release drug delivery systems is therefore important when developing such novel treatments for OME and biofilms.
2.5 MODIFIED RELEASE DRUG DELIVERY SYSTEMS

As eradication of biofilms is likely to require high-dose locally-delivered antibiotics over a prolonged time period, the use of a modified-release drug delivery system may be one way of achieving that aim. Although modified-release drug delivery systems have only been developed over the last few decades, they have resulted in great improvements in patient care. The increase in the use of modified-release drug delivery systems can be attributed to the major advantages they offer over conventional drug delivery (Anelli 2008) (Bajpai, et al. 2010) (Rosoff 1989) (Siepmann and Siepmann 2008a), including:

- Regulation of drug concentrations over a specific time period
- Reduced administration frequency
- Accessing sites of therapeutic interest that cannot be practically accessed using standard delivery (e.g. inside the blood brain barrier)
- Drug targeting, with drugs being delivered only to the site where it is most needed
- Pulsatile drug delivery, where drug is delivered at set time points, or pulsatile dosing with several drugs released at different lag periods (chronotherapeutics)
- Potential for development of intelligent drug delivery systems that release drugs in response to a pre-defined biological event / parameter
2.5.1 Modified drug delivery systems

To control the rate at which drugs are released (Siepmann and Siepmann 2008a), the active ingredient may be embedded in a matrix of some type. Biodegradable polymers (large molecules composed of repeating structural units) are often used for this purpose. Three broad types of drug delivery systems are associated with biodegradable devices, shown in Fig 2.5.1.1, although in reality many devices' drug release is governed by a combination of factors.

In diffusion-controlled systems the drug diffuses down a concentration gradient from the delivery device into the surrounding area. Different types of diffusion can be distinguished (Morishita and Park 2010):

- Reservoir devices with a core-shell structure: Drug is at the centre of a shell, and diffuses through shell at a controlled rate (either with constant or non-constant activity)
- Matrix or monolithic devices: Drug is distributed homogenously throughout the system
- Laminated matrix devices contain a mix of matrix and reservoir devices with characteristics of both.
Fig 2.5.1.1: Schematic illustration of different patterns of drug release (Rosoff 1989).
In all three cases the drug can exist in the system as a solution (drug molecularly distributed within system) or a dispersion. Matrix systems can be divided into those that are based on hydrophobic and those based on hydrophilic rate-controlling materials (Li and Jasti 2006). The hydrophobic systems keep their shape, with release of the active ingredient occurring by diffusion. The hydrophilic matrix devices swell in contact with an aqueous solution, with the control of release then affected by both diffusion and swelling, as well as matrix degradation (Ranade and Hollinger 2003).

In swelling-controlled systems, drug release from the matrix occurs as the swelling front develops and moves into the release device. As the water reaches the inner core of the polymer matrix, drug is released into the aqueous phase and then to the surrounding fluid. In addition to matrix devices, hydrogels also release drugs in this way. Hydrogels are water-insoluble hydrophilic polymers that swell in water (Bajpai, et al. 2010), and the drug release from the hydrogel occurs when the drug comes into contact with the water front moving into the gel.

Polymer swelling is also of importance, as it increases the length of the diffusion pathways, and therefore reduces drug concentration gradients and release rate. On the other hand, swelling can increase polymer molecular mobility and thus drug mobility, increasing the drug release rate.

Erosion refers to the process of material loss from the polymer bulk, in contrast to polymer degradation that refers to a chain scission process by
which polymers are cleaved into oligomers and finally monomers. Erosion is classified into surface and bulk erosion. In surface erosion degradation occurs primarily in the outermost layers, so that as the surface polymer degrades the drug is freed; this results in a gradual shrinkage of the device and leads to erosion-controlled drug release. This surface erosion is distinct from bulk erosion, where the entire device is rapidly penetrated by water and thus polymer chains are cleaved throughout the system. Thus in bulk erosion the porosity increases, embedded molecules become more mobile and diffuse out of the system, the device outer dimensions stay the same until eventually the device disintegrates into smaller polymer fragments (Hausberger and DeLuca 1995). Polymers with very reactive functional groups degrade fast with surface erosion, whereas polymers with less reactive groups (such as PLGA) undergo bulk erosion. Thus surface erosion is like a bar of soap that gradually reduces in size, and erosion controls drug release, but bulk erosion is like a biscuit that crumbles when wet and drug release is governed by diffusion. Despite this classification, in reality the exact mode of erosion is affected by the balance between system wetting rate and polymer cleavage rate, the former also being affected by the dimensions of device (smaller devices are more readily wetted than larger ones) (von Burkersroda, Schedl and Gopferich 2002).

In practice, several different mass transport phenomena concurrently affect drug release from modified-release systems (Siepmann and Siepmann 2008a) (Siepmann and Siepmann 2008b). In the case of PLGA systems, water penetration into the device is faster than polymer chain cleavage by ester hydrolysis and the system undergoes bulk erosion. This generates short chain
acids within the device, which diffuse out of the device into the surrounding fluid. Although bases can diffuse from the surrounding into the system to neutralise the short chain acids within the device, their diffusion can be relatively slow leading to a pH drop in the core of the device (Brunner, Mader and Gopferich 1999). This can accelerate polymer degradation and drug release, and may lead to undesirably rapid release of a dose of drug that was meant to be released over a slower time period (dose dumping) (Anelli 2008).

Other factors affecting drug release (Li and Jasti 2006) (Morishita and Park 2010) include solubility of drug in the device and surrounding medium, concentration gradient, drug loading, morphological characteristics such as porosity and surface area, hydrophilicity and hydrophobicity of the system, chemical interaction between drug and polymer, polymer characteristics such as molecular weight and glass transition temperature. For example, a high molecular weight drug will diffuse more slowly, binding effects will slow down the diffusion, a large molecular weight polymer will degrade more slowly, a hydrophobic polymer will absorb less water leading to less bulk degradation, and smaller more porous particles will have a greater total surface area with consequent faster diffusion of solvent into the particles and faster matrix degradation rate (Morishita and Park 2010).

2.5.2 Biodegradable drug delivery systems
Although drugs can be delivered in a controlled manner using non-biodegradable vessels, biodegradable systems have significant advantages as the need for vessel removal is avoided (Bossy, et al. 2008) (Li and Jasti
A variety of polymers can be used, including poly(amide), poly(esters), poly(orthoesters), poly(anhydrides) and others (Rosoff 1989). Poly(esters) particularly have been widely used, as they break down to naturally occurring substances, degradation only requires water, a wide variety of different properties can be obtained depending on monomers chosen, and early experience with suture materials means that a wealth of safety data already exists (Saltzman 2001).

PLGA (Fig 2.5.2.1), a poly(ester) polymer has been the most widely studied biodegradable system (Bossy, et al. 2008) (Lu, et al. 2009), and has a long history of safe clinical use in sutures, implants, prosthetic devices, and depot drug delivery systems (e.g. Zoladex). It is biodegradable and biocompatible and approved by the US Food and Drug Administration. PLGA is synthesized by ring opening and co-polymerisation of two different monomers, lactic acid \((C_3H_6O_3)\) and glycolic acid \((C_2H_4O_3)\), linked by an ester linkage. The result is a polymer, a large molecule composed of repeating structural units. The precise form is poly(DL-lactic acid-co-glycolic acid), the DL showing that it is the racemic mixture of lactic acid (containing both stereoisomers). The polymer forms amorphous structures, glass-like with tangled chains (as opposed to ordered crystalline structures), and application of heat turns brittle solid material to a viscous liquid. Degradation involves hydrolysis in the presence of water, into monomers of lactic and glycolic acids, which are metabolised without any adverse reactions. Different forms of PLGA are characterised by different ratios of lactic to glycolic acids.
Fig 2.5.2.1: Poly(lactic-co-glycolic acid); x = number of units of lactic acid, y = number of units of glycolic acid.

Drug release is typically triphasic, an initial burst due to release of drug on the surface and in the water channels, a second stage where drug is released by diffusion from the polymer, and a final burst as the polymer degrades (Yasukawa, et al. 2001). It is desirable for the second stage to exhibit zero order kinetics, where the amount of drug released from the device is independent of the amount of drug in the device, in other words an identical amount of drug is released during each time period (Bruck 1983). The general factors discussed above affect drug release, but there are also some issues specific to PLGA. Although the 50:50 form of PLGA has the fastest degradation time (about two months), generally a higher lactic acid content delays degradation, as does higher molecular weight. High drug loading may also increase release rate, but this may largely be due to the initial burst rather than sustained release. Blending different polymers can achieve drug delivery for up to a year whilst still remaining biodegradable (Kunou, et al. 2000).
PLGA can be used to form a matrix system with the drug dispersed between PLGA microparticles, or the drug can be encapsulated within each PLGA microsphere in the form of a suspension, liquid or solid (Fig 2.5.2.2).

Fig 2.5.2.2: Drug (red) can be dispersed between PLGA particles (blue), or encapsulated within PLGA

Commercially available alternatives to PLGA (Lane, Okumu and Balasubramanian 2008) include microspheres and injectable gels, such as ReGel depot technology (PEG and PLG polymer) and the Atrigel drug delivery system. Biodegradable nanoparticles and liposomes can be injected intravenously with the intention of localising the drug to one particular area, but with these systems in addition to drug release itself, drug localisation and potential toxicity in non-target tissues also requires investigation. Modification of the drug itself can also enhance drug delivery (Ayoub, Wedemeyer and Wohr 2008), for example a pro-drug may be converted to active drug at site of interest, and addition of polyethylene glycol (PEG) to a drug (PEGylation, an FDA approved technology) increases its half life. PEG is eliminated by renal excretion. Delivery of a drug local to its intended site of action is a particularly
effective strategy, as often therapeutic effect can be achieved with minimal risk of systemic adverse reactions (Costantino, et al. 2007). The availability of injectable formulations that aggregate at body temperature has further expanded the potential applicability of novel drug delivery techniques (Fraylich, et al. 2008).

2.5.3 Novel drug delivery to eradicate biofilms

The application of novel drug delivery to the treatment of biofilms is a logical evolution for the multi-disciplinary translational researcher. Modification of implantable materials (Bayston, Ashraf and Bhundia 2004) (Bayston, Fisher and Weber 2009) (Biedlingmaier, Samaranayaki and Whelan 1998) (Furno, et al. 2004) or the use of ultrasound (Ensing, et al. 2006) (Huang, et al. 1996) or low-strength electrical fields (Blenkinsopp, Khoury and Costerton 1992) has already been mentioned, but the latter two techniques cannot be applied to the middle ear where neural structures and fluid filled cavities of the inner ear are in close proximity, nor is there access for photodynamic therapy. Eradication of biofilms is challenging (Lewis 2001), but biodegradable polymers may be a useful future strategy.

Biodegradable polymers have been identified as a potential method of delivering antibiotics in a modified release manner (Cheow, Chang and Hadinoto 2010) (Coowanitwong, et al. 2008) (Smith 2005), with particular interest in the field of infected orthopaedic implants. However, within the middle ear, some studies of using modified drug delivery to eradicate middle ear infections have also been published. Goycoolea et al (M. Goycoolea, D.
Muchow, et al. 1991) (M. Goycoolea, D. Muchow, et al. 1992) (Goycoolea and Muchow 1994) studied modified release ampicillin from a poly-L-lactic acid support in cat and chinchillas animal models, and found the method was effective at eradicating infection without any ototoxicity. Whilst encouraging, this is not a true representation of human OME, as animal models are either acute infections or Eustachian tube obstructions and no true animal model of OME exists. The important link with biofilms was also not investigated. Another study using pluronic F-127 to deliver vancomycin as a treatment of experimentally induced MRSA middle ear infection (Le, et al. 2004) is again encouraging but not a true representation of human OME.

A major advantage of a modified release system to eradicate biofilms is the ability to place the device at the site of action. It has already been mentioned that biofilm eradication requires a locally high antibiotic dose, which would be difficult to achieve with systemic administration without major systemic toxicity; the relationship between minimum effective concentration and minimum toxic concentration is described by the therapeutic window (Fig 2.5.3.1). Alternatives to placing antibiotics locally at site of action would be systemic administration of an agent that concentrates at a specific site, such as liposomes. These are composed of a phospholipid membrane surrounding an aqueous core that can hold therapeutic agents; they can be used to target drugs (Smith 2005) to phagocytes and the reticulo-endothelial system, but if coated with a surface antibody can be targeted to specific bacterial biofilms (Robinson, Creeth and Jones 2000).
Advances in the field of drug delivery are likely to provide crucial new ways of eradicating biofilms. We chose to use a PLGA based monolithic device for the experiments on biofilm eradication as a novel treatment for OME, combined with a gel, either poloxamer (Fig 2.5.3.2) or carboxymethylcellulose (Fig 2.5.3.3), plus rifampicin and clindamycin. The rationale for the choice of this modified release formulation, and its advantages and disadvantages, is discussed more in Chapter 11.

\[ H(OCH2CH2)_{x}(OCH2CH)_{y}(OCH2CH2)_{z}OH \]

Fig 2.5.3.2: Poloxamer (Sigma 2012)
Fig 2.5.3.3: Sodium carboxymethylcellulose (Sigma 2012)
2.6 SUMMARY

Glue ear is an extremely common condition, with VTs insertion being one of the commonest surgical procedures. Whilst the recent NICE guidelines on the surgical management of OME have clarified best practice, it is not yet known whether they have had an impact on OME treatment, nor whether there is a variation in compliance across different hospitals. A great degree of controversy surrounds adjuvant adenoidectomy in conjunction with VTs to try and reduce the high rate of further surgery after VT extrusion, leading to the setting up of a multicentre UK study on glue ear treatment. The MRC Trial of Alternative Regimens on Glue ear Treatment (TARGET) examined children up to 2 years after randomisation to VTs, VTs+ad or watchful waiting, finding in favour of adjuvant adenoidectomy. However, with OME's chronic course, longer-term data is also of value, warranting supplementary studies beyond the TARGET 2 years.

The aetiology of OME is unclear, but the role of bacteria and biofilms warrants further investigation using new methods that can demonstrate bacteria in the middle ear effusion. If OME is a biofilm infection, it is not surprising that VT insertion has such a high need for further surgery; VTs only remove the effusion, but do not deal with the underlying problem of a biofilm infection. A new treatment strategy based on better understanding of OME pathogenesis is therefore required.
In order to study OME biofilms and their eradication in the laboratory setting, a new \textit{in vitro} model is required. Demonstration of biofilm structure as well as its functional characteristics, reduced susceptibility to antibiotics, will have to be demonstrated. High dose antibiotics delivered locally over several weeks are likely to be required for biofilm eradication, and the new model must be able to incorporate the addition of antibiotics or a modified release device. Advances in the field of drug delivery are likely to provide crucial new ways of eradicating biofilms.
3 AIMS

The thesis has two distinct parts: a collection of clinical studies, and series of laboratory experiments. The aims are as follows.

CLINICAL STUDIES

- Assess need for further surgery for OME after VT extrusion (TARGET trial long-term data)
- Assess the impact of adjuvant adenoidectomy on need for repeat surgery (TARGET trial long-term data)
- Assess practice in accordance with NICE guidelines on OME management, including whether they have changed our practice

LABORATORY STUDIES

- Consolidate existing microbiology data obtained by our group and establish presence of bacteria and biofilms in middle ear effusions
- Develop an in vitro model to represent S. aureus biofilm infection
- Assess whether antibiotics (rifampicin and clindamycin) can be used to eradicate S. aureus biofilms using the model
• Produce a biodegradable modified-release antibiotic pellet based on a PLGA matrix with rifampicin and clindamycin, and with either pluronic F127 or carboxymethylcellulose gels

• Assess antibiotic release from the pellets with High Performance Liquid Chromatography (necessitating a new method) and Serial Plate Transfer Testing

• Test the antibiotic pellets against biofilms in the in vitro model
4 THESIS OUTLINE

The thesis is split into chapters that address specific questions. Each chapter contains a brief introduction summarising the important issues discussed in the main introduction (Chapter 2), a description of methods, followed by results and comprehensive discussion.

The clinical chapters demonstrate that guidelines on OME treatment are not always followed, and that current OME treatments have major shortcomings, with many children requiring VT surgery more than once. Additional adenoidectomy may improve on this, but the rate of recurrence remains high. Thus there is a clear need to develop better treatments, and better understanding of OME aetiology is key to developing new therapeutic strategies. Therefore, the role of bacteria and biofilms in OME is investigated next, demonstrating the important role that these play in the aetiopathogenesis of OME.

In order to study the treatment of biofilms, an in vitro S aureus biofilm model is developed next, and used to test whether rifampicin and clindamycin can be used to eradicate biofilms. Demonstrating that they can, a modified-release antibiotic PLGA-based formulation is then developed. Drug release from these antibiotic pellets is investigated with High Performance Liquid
Chromatography and Serial Plate Transfer Testing, and the pellets tested in the *in vitro* biofilm model showing that they are capable of eradicating biofilms.

Overall, the thesis shows that current treatment has significant shortcomings, but better understanding of OME aetiopathogenesis opens up the possibility of improved treatment. Although further development is required, antibiotic pellets may be a useful, better future treatment for OME.
5 CURRENT OME TREATMENT: VENTILATIONS TUBES AND
THE BENEFITS OF ADENOIDECTOMY

5.1 INTRODUCTION

Examination of the effectiveness of current OME treatment is the starting point for this thesis, underpinning the need for further investigation into OME aetiology and the development of improved treatment based on better understanding of OME pathogenesis. As described in the introduction, current treatment guided by NICE is Ventilation Tube (VT) insertion. However, about a quarter of children are thought to require further surgical treatment, although adenoidectomy may reduce this (Gates, Avery and Prihoda 1987) (Kadhim, et al. 2007) (Maw and Bawden 1994) (Popova, Varbanova and Popov 2011) (Rosenfeldt and Bluestone 1999).

The UK Trial of Alternative Regimens in Glue Ear Treatment (TARGET) set out to determine how effective VTs were and what was the role of adjuvant adenoidectomy. TARGET was a multicentre trial, examining children up to 2 years after randomisation to VTs, VTs and adenoidectomy (VTs+ad) or further watchful waiting (the term further was used because all children had already undergone a three month observation period prior to randomisation). There were also some children in whom symptoms were deemed so severe that withholding surgery would be unethical (termed obliged to treat, OTT), and these were listed straight for surgery, with the type of surgery allocated being
chosen by the clinician on the basis of need; these children were included in the final analysis together with the randomised ones to boost patient numbers.

VTs were shown to benefit hearing, but only for as long as they stay in place (Browning 2012) (MRC Multicentre Otitis Media Study Group 2001a) (MRC Multicentre Otitis Media Study Group 2001b). Once VTs extruded, those children treated with VTs had hearing that was the same as the children receiving no surgery; a variety of reasons was responsible for this lack of a benefit of VTs at 2 years, including the fact that in the VT group hearing again deteriorated after VT extrusion, in the further watchful waiting group hearing improves over time in keeping with natural history of OME, and also because 62% of the group randomised to further watchful waiting actually switched to receive surgery within a year (thus diminishing benefits of surgery if analysis on intention to treat basis is performed; further watchful waiting was incorporated into TARGET to determine if surgery could be avoided altogether, but the high switching rate from observation to surgery confirms parental and clinician beliefs that surgery is required and effective).

In contrast, adjuvant adenoidectomy benefits hearing for the full 2 year duration of the trial, so that even after VTs have extruded the children that had VTs+ad still have better hearing than those receiving no surgery (Fig 5.1.1).
Fig 5.1.1: Hearing results from TARGET (Courtesy of Mark Haggard), showing that hearing improves in further watchful waiting group, but deteriorates 12 months after VTs so that the hearing loss in the two groups at 12 months is comparable. In contrast, children receiving VTs+ad have better hearing at 24 months that either of the two other groups.

TARGET only examined children over a two year period. However, as already detailed in the introduction, the natural history of OME often spans years, with 20-25% thought to need surgery more than once. Therefore, a follow-up period of more than 2 years post-surgery is clearly desirable.

The work presented here therefore represents a longer follow-up period (minimum 7.5 years from randomisation) on a subset of TARGET children (those enrolled in Leicester), and examines need for revision surgery and
follow-up visits after VT insertion. The effect of adding adenoidectomy to VTs is also assessed; comparison is made between those children that had VTs alone and those that had VTs+ad, but because of high switching to surgery, the further watchful waiting group is not analysed as a group in its own right. In addition to examining current outcomes, this chapter comments on the appropriate time scale for evaluation of treatment in a potential future clinical trial.
5.2 METHODS

TARGET, the Trial of Alternative Regimens in Glue Ear Treatment has been described above, and its structure and patient flow are shown in Fig 5.2.1 and 5.2.2, respectively. Children aged 3.5 to 7 years, with a hearing level of 20 dB NHL or worse, and tympanometry indicative of middle ear fluid (B on both sides, or B on one side and C2 on the other), were included. The "obliged to treat" children were included with the randomised ones to boost numbers for analysis, with work by TARGET team confirming validity of this strategy instead of analysis by intention to treat, because both analysis strategies yield very similar results; the combined analysis was termed "as allocated" as it was performed on the basis of a treatment that the child was allocated rather than randomised to.

In addition to short (2 years) scheduled trial follow-up, TARGET obtained outcomes in an adjunct study after visit 7 (post visit 7, PV7, study) that examined the need for further treatment after the scheduled two years of the initial trial. However, the PV7 length of follow-up was 1.2-5.5 years after trial exit, meaning that the follow-up in some cases was as short as 3 years and 3 months from randomisation. Therefore, an additional way of assessing long term outcomes was required. The original TARGET data was collected by the TARGET multi-centre team, with my involvement dealing purely with this supplemental, long-term study shown in this thesis.
The data presented in this chapter encompass a further adjunct study, TARGET long term, which analysed consultations even beyond PV7 study, although only on a subset of patients. The minimum follow-up period was 7.5 years from randomisation to the time of notes review taking place. TARGET long term study assessed effectiveness of VTs and VTs+ad by analysing the number of follow-up visits required and any need for further surgery. The notes of Leicester TARGET children were examined, and data on clinic visits and further surgery collated with existing information from the original two-year trial and the subsequent PV7 study. The TARGET team confirmed that Leicester children were representative of the whole TARGET group.

Two different types of analysis were performed to compare VTs with VTs+ad. The first analysed children on the basis of what treatment they were allocated (encompassing the randomised children, as well as the obliged to treat ones). The second analysed children on the basis of what treatment they actually received, and thus contains a large number of children that were initially allocated further watchful waiting but switched to a surgical arm. Overall patient numbers are relatively small; for example, if one were to look for rates of repeat surgery to fall from 30 to 15%, a total of 124 patients in each group would be required to have 80% power to detect a significant difference at the 0.05 level (using Lehr’s formula). Statistical analysis was performed using SPSS PASW 18.
3 month active observation for those meeting criteria at first visit

Visit 1
Inclusion criteria:
- 3.5-7 yrs old
- better ear ≥20dB
- B/B or B/C2 tympanograms

OTT at visit 1

Visit 2
RANDOMISATION
for all requalifying

OTT at visit 2

FWW VTs VTs+ad

2 yrs F-U

EXIT: visit 7

PV7 TLT

Fig 5.2.1: Structure of TARGET and supplementary studies. PV7: post-visit 7 study. TLT: TARGET long term study. OTT: Obliged to treat
Fig 5.2.2: Patient flow in TARGET clinical trial. ( Courtesy of Mark Haggard and Josephine Higson)
The following variables were examined:

- Number of clinic visits at any time from randomisation
- Number of clinic visits after the final planned trial visit at 2 years after randomisation
- Number of OME-related surgical procedures at any time from randomisation
- Number of OME-related surgical procedures after the final planned trial visit at 2 years after randomisation
- Number of patients having OME-related surgery in addition to the treatment that they were allocated (in case of “as-allocated” analysis),
- Number of patients having more than one procedure (in case of “as-treated” analysis)
- Length of clinical care, from randomisation to last clinical encounter
- The percentage of patients visiting clinic after the trial end date
- The percentage of patients having OME-related surgery after the trial 2 years
- The percentage of patients visiting clinic or having OME-related surgery after the trial 2 years

Only OME-related additional procedures (VTs or other middle ear surgery, adenoidectomies) were counted.

To assess how long any future trial should last, the number of visits made in each subsequent year after the end of the trial 2 years was examined.
5.3 RESULTS

Of the 432 randomised plus obliged to treat children in TARGET, 102 were enrolled in Leicester (almost one quarter of the total): 93 randomised and 9 obliged to treat. Medical records of 98 children were available for analysis. The minimum follow-up period from randomisation was 7.5 years, ranging up to 11.6 years.

Table 5.3.1 shows the outcomes when patients were analysed on the basis of what treatment they were allocated (encompassing those that were randomised plus obliged to treat children). Table 5.3.2 compares outcomes when analysis was performed on the basis of what the patient actually received (and thus contains additional switchers from further watchful waiting to surgery).
<table>
<thead>
<tr>
<th>ANALYSIS: AS-ALLOCATED</th>
<th>VTs (N=33)</th>
<th>VTs+ad (N=35)</th>
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<tr>
<td><strong>CLINIC VISITS</strong></td>
<td></td>
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<tr>
<td>Mean N of visits at any time (SD)</td>
<td>9.58(5.62)</td>
<td>8.46(4.84)</td>
<td>0.381</td>
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<tr>
<td>Mean N of visits after last trial visit (SD)</td>
<td>3.79(5.22)</td>
<td>2.57(4.05)</td>
<td>0.285</td>
</tr>
<tr>
<td><strong>SURGICAL PROCEDURES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean N of procedures per patient at any time (SD)</td>
<td>2.27(1.35)</td>
<td>1.66(1.06)</td>
<td>0.040</td>
</tr>
<tr>
<td>Mean N of procedures after last trial visit (SD)</td>
<td>0.85(1.09)</td>
<td>0.49(0.85)</td>
<td>0.131</td>
</tr>
<tr>
<td>N (%) pts having procedures additional to allocation</td>
<td>21 (63.6)</td>
<td>16(45.7)</td>
<td>0.138</td>
</tr>
<tr>
<td><strong>LENGTH OF CARE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years (SD)</td>
<td>4.25(2.73)</td>
<td>3.62(2.69)</td>
<td>0.334</td>
</tr>
<tr>
<td><strong>NEED FOR CARE AFTER LAST TRIAL VISIT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%) of patients attending clinic</td>
<td>24(72.7)</td>
<td>18(51.4)</td>
<td>0.071</td>
</tr>
<tr>
<td>N (%) of patients having surgery</td>
<td>18(54.5)</td>
<td>11(31.4)</td>
<td>0.054</td>
</tr>
<tr>
<td>N (%) of patients attending clinic or surgery</td>
<td>24(72.7)</td>
<td>19(54.3)</td>
<td>0.115</td>
</tr>
</tbody>
</table>

Table 5.3.1: TARGET Long Term outcomes, analysing patients on the basis of allocated treatment. N: number. SD: standard deviation. P: p-value obtained using Chi squared test (comparing proportions) or t-test (comparing means).
### ANALYSIS: AS-TREATED

<table>
<thead>
<tr>
<th></th>
<th>VTs (N=40)</th>
<th>VTs+ad (N=46)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLINIC VISITS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean N of visits at any time (SD)</td>
<td>9.90(5.40)</td>
<td>8.65(4.56)</td>
<td>0.250</td>
</tr>
<tr>
<td>Mean N of visits after last trial visit (SD)</td>
<td>4.05(5.09)</td>
<td>2.70(3.85)</td>
<td>0.165</td>
</tr>
<tr>
<td>SURGICAL PROCEDURES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean N of procedures per patient at any time (SD)</td>
<td>2.23(1.31)</td>
<td>1.65(0.97)</td>
<td>0.023</td>
</tr>
<tr>
<td>Mean N of procedures after last trial visit (SD)</td>
<td>0.88(1.09)</td>
<td>0.50(0.86)</td>
<td>0.079</td>
</tr>
<tr>
<td>N (%) pts having more than one procedure</td>
<td>28(70.0)</td>
<td>27(58.7)</td>
<td>0.276</td>
</tr>
<tr>
<td>LENGTH OF CARE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years (SD)</td>
<td>4.45(2.80)</td>
<td>3.73(2.61)</td>
<td>0.223</td>
</tr>
<tr>
<td>NEED FOR CARE AFTER LAST TRIAL VISIT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%) of patients attending clinic</td>
<td>30(75.0)</td>
<td>26(56.5)</td>
<td>0.073</td>
</tr>
<tr>
<td>N (%) of patients having surgery</td>
<td>22(55.0)</td>
<td>15(32.6)</td>
<td>0.036</td>
</tr>
<tr>
<td>N (%) of patients attending clinic or surgery</td>
<td>30(75.0)</td>
<td>27(58.7)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

Table 5.3.2: TARGET Long Term outcomes, analysing patients on the basis of actual treatment received. N: number. SD: standard deviation. P: p-value obtained using Chi squared test (comparing proportions) or t-test (comparing means).

The tables show that, whether analysed "as-allocated" or "as-treated", this group of children, entering TARGET with hearing loss of at least 20 dB NHL lasting at least 3 months, have significant healthcare needs during the trial and also afterwards. Patients allocated to VTs make nearly 4 clinic visits after...
the trial ended, and their clinical care lasts on average 4.25 years from randomisation. Nearly three quarters needed to attend either clinic or have surgery even after the trial 2 years.

In terms of need for revisions surgery, 63.6% of those allocated VTs end up having more surgery, and 70% of those receiving VTs as their first procedure have surgery again. Adenoidectomy does not significantly reduce need for revision surgery, but does appear to reduce the mean number of procedures (at any time) per patient, from 2.27 and 2.23 amongst those allocated and receiving VTs, respectively, to 1.66 and 1.65, respectively (p=0.040 and 0.023).

The number of visits made in each 6 month period following trial end is shown in Fig 5.3.1. Three quarters of children receiving VTs made clinic visits or needed surgery after trial 2 years, and it can be seen that the majority of visits after the 2 trial years happen within the first few years, as might be expected.
Fig 5.3.1: Total number of visits taking place during each 6 month period following TARGET 2 years.

The number of visits (following the end of the 2 year trial) made by children of different ages is shown in Fig 5.3.2. The mean age of children making visits after trial ended was 9.4 years, range 6.0 to 17.0, with mode 7.0 and median 9.0. After 11 years of age, the number of visits falls off, and more than 95% of visits have happened before 14 years of age. Fig 5.3.3 details the age at which last visit was made by each of the patients. The mean age at the last visit was 8.8 years, ranging from 4.4 to 17.1, with a mode of 13.0 years and median on 8 years. Majority of patients were aged 7-9 when they last made their ENT visit, and a further steep fall can be seen after 13 years. Taking these two last measures together, it would appear that by the time a child reaches 14 years, majority of OME-related disease appears to have resolved.
Fig 5.3.2: Frequency distribution of the ages of patients making visits after the end of trial 2 years.
Fig 5.3.3: Frequency distribution showing the patients' ages at last clinic visit.
5.4 DISCUSSION

5.4.1 Summary of results

The research presented here examined long term (>7.5 years from randomisation) outcomes of VTs and VTs+ad, on a subset of children enrolled in the 2-year TARGET randomised controlled study of children with OME lasting at least 3 months and with a hearing loss of at least 20 dB NHL. Children allocated to VTs make on average nearly 4 clinic visits after the trial ended, their clinical care lasts on average 4.25 years from randomisation (and the time waiting for randomisation, initial ENT visit, and GP care on top) and nearly three quarters still needed to either attend clinic or have surgery even after the trial 2 years. Repeat surgery is necessary in 63.6% of those allocated VTs, and 70% of those that received VTs as their first treatment. Adenoidectomy did not significantly reduce need for revision surgery, but did reduce the mean number of procedures per patient from 2.27 to 1.66 (as-allocated) and from 2.23 to 1.65 (as-treated).

5.4.2 Choice of study population

A major advantage of the TARGET clinical trial was that it studied the severely affected children that are being considered for surgery in modern UK practice; in fact, the TARGET entry criteria have now been broadly adopted by the NICE guidelines as the criteria defining eligibility for surgical treatment (NICE 2008). Therefore, the TARGET children are the ones that have severe disease, and are representative of the kind of children that are treated in UK today.
The TARGET children also represent a much more severe population than has been studied by some other trials that may have included children with unilateral OME or OME of short duration, therefore representing a population of children in whom OME is likely to resolve soon anyway; for example, the Paradise trial that is often quoted as saying that VTs are "ineffective" included children with unilateral effusions or OME that is not continuously present for a minimum of 3 months, and in fact only 20% of the children studied had bilateral OME continuously for a minimum of 3 months (Paradise, et al. 2007). The fact that over half of those allocated in TARGET to a non-surgical arm switched to surgery supports the severity of OME in the population studied, as does the very high need for repeat surgery (63.6% in VTs and 45.7% in VTs+ad), and the high number of mean procedures per patient (2.27 and 1.66, respectively). The use of short term ventilation tubes (Shepard tubes) in the trial is likely to be partly responsible for the high revision surgery rate; in addition, the longer one follows patients the more one sees need for repeat surgery, so that studies with > 2 years follow-up typically report much higher revision surgery rates (68% (Maw and Bawden 1994) and 36% (Kadhim, et al. 2007)) that are also seen in TARGET Long Term study. The fact that usual figures quoted for repeat surgery are 20-25% (Gates, Avery and Prihoda 1987) again illustrates that TARGET studied a severely affected population and that long follow-up is imperative.
5.4.3 Choice of outcome measure

Defining "success" of OME treatment is difficult. Hearing levels are easy to measure, but in themselves do not necessarily reflect the complex interaction between hearing, child's personality and abilities, environment, and adult input, all of which together influence a child's performance in their day to day activities, schooling and speech. Much research has looked at hearing levels because this is a quantitative variable that surgeons understand, but the "softer" outcomes have been less well studied.

Looking at need for surgery or clinic visits as an outcome measure has distinct advantages. Both are determined by a composite that includes all the different variables that interact to affect how a child and parents deal with hearing loss, from the actual hearing level through fluctuation, intellect and social environment. However, such outcome measures are not objective like hearing levels or tympanograms, because they are influenced by parental and surgeon preferences; nevertheless, they are useful because they represent what happens in the real world. They are also very practical when exploring long term outcomes, as they simply record standard practice, and no additional investigations are required. For example, a child that is well is unlikely to wish to return for a hearing test 5 years after surgery because of trial requirements, but if they are no longer being seen in clinic it is reasonable to assume that they are having no or minimal problems.
However, a strategy based on hospital records follow-up does not take account of any care given in the community for example by the General Practitioners, and it also relies on people not visiting other hospitals or moving away. These concerns cannot be answered by our study, but it is unlikely that children randomised to one treatment are more likely to move away than those randomised to another arm, and only one hospital in Leicester provides ENT services. So, subject to reservations outlined above, looking at clinic visits and need for further surgery is a reasonable and practical way of examining long-term outcomes of OME treatment.

5.4.4 Treatment with VTs
TARGET showed that during the 2 years of the trial, VTs conferred a hearing benefit compared with no surgery. However, this was short lived, likely because hearing deteriorates once tubes extrude, those in no surgery group may spontaneously improve, and also the switching from further watchful waiting into surgery diminishes the benefits seen with VTs. In terms of hearing, at 2 years there is little difference between children randomised to VTs or no surgery.

TARGET long term study assessed clinic visits and need for further surgery. In terms of clinic visits, any that occurred after the trial 2 years are more informative than those within 2 years, because the trial itself called for regular scheduled visits, but any attendances after 2 years represent genuine clinical need.
The results demonstrate that the clinical needs of children receiving VTs are substantial and stretch well beyond two years post randomisation. Analysing children as-treated shows that the mean number of visits was 9.9 (this also encompasses the 5 visits routinely planned as part of TARGET), but 75.0% of children still made clinic visits after trial ended, with the mean number of visits following trial end being 4.1. Also, 70.0% had to have surgery again, the mean number of procedures per patient was 2.2, and the mean length of care from randomisation to last recorded visit was 4.5 years.

The need for further surgery in TARGET long term study appeared to be higher than the 20-25% rate reported in other studies (Gates, Avery and Prihoda 1987). As discussed above, a longer follow-up period picks up more children with on going problems, so that other studies looking at long term outcomes do typically report higher need for revision surgery, for example 36 - 68% (Kadhim, et al. 2007) (Maw and Bawden 1994). Additionally, the TARGET children represent a severely affected population and as such would be expected to have worse outcomes that the mildly affected children studied elsewhere (Paradise, et al. 2007). TARGET also used short term ventilation tubes (Shepard tubes), and although such tubes are less likely to lead to persistent perforation once tube extrudes, the fact that they only stay in for 6-9 months or so means that many children remain troubled by OME once tubes extrude.

The data on VT outcomes presented here is also in keeping with studies examining spontaneous OME resolution without surgery, which demonstrated
that, in children with bilateral OME that has already persisted for 3 months at the start of the study, OME has resolved in only a third of children 1-2 years later (Introduction Table 2.2.3.1) (Rosenfeldt and Bluestone 1999) (Maw and Bawden 1994). This is in keeping with TARGET’s findings and the suggestion that VTs are a temporary management solution while waiting for OME to spontaneously resolve.

Given the high need for repeat surgery following VTs, a treatment that would provide a more long-term or permanent solution for OME would be welcome.

5.4.5 Adding adenoidectomy to VTs

The TARGET 2 years had already shown that adenoidectomy in addition to VTs benefits hearing during and at the end of the trial 2 years. Adenoidectomy also appears beneficial later on as shown here, in terms of a reduction in the mean number of procedures per patient, although the magnitude of benefit (approximately 0.6 procedures per patient less with adenoidectomy) may not be large. Small patient numbers make meaningful statistical interpretation difficult when examining recurrence rates, and a larger patient group would be required to show a definitive benefit (or lack thereof). Adenoidectomy may also confer other benefits, for example less nasal obstruction, snoring and sleep apnoea, and sinusitis (Lieser and Derkay 2005) (Helfaer, et al. 1996), but these have not been studied here.

One could argue that the reason that children allocated VTs alone end up having more procedures is simply because they still have the adenoid that
may end up being removed for non-OME reasons. However, all but one adenoidectomy in this study was performed in conjunction with ear surgery; one child had adenoidectomy together with tonsillectomy as a second procedure, but then ended up having revision adenoidectomy and VTs as a third procedure. Therefore, it cannot be said that adenoidectomies for non-OME reasons form a significant number of cases. Additionally, because the study allowed the treatment of children on an "obliged to treat basis", any child with adenoid-related symptoms right at the start would not have been included in randomisation but instead would be in the obliged to treat group that ends up being analysed in the VTs+ad group. Whilst children may of course develop adenoid-related symptoms after randomisation, at least the ones with adenoid symptoms at the start are likely to have ended up with adenoidectomy as their primary treatment.

Other research has also found adenoidectomy to be beneficial, as discussed in the main introduction, finding reduced time with effusion, reducing the mean number of tubes per patient, and a halving of the need for further surgery (Black, Sanderson, et al. 1990) (Gates, Avery and Prihoda 1987) (Kadhim, et al. 2007) (Maw and Bawden 1994) (NHS Centre for Reviews and Dissemination 1992) (Popova, Varbanova and Popov 2011) (van den Aardweg, Schilder, et al. 2010), although this has not been found universally (Casselbrant, Mandel, et al. 2009) (Dempster, Browning and Gatehouse 1993). As discussed in main introduction, the likely reasons for adenoidectomy being beneficial to OME is the removal of nasopharyngeal biofilms that can act as a persistent focus for infection ascending the

Despite strong evidence in favour of adding adenoidectomy to VTs, this is not the recommended practice. UK guidelines (NICE 2008) advise that adenoidectomy should be performed if there are other, non-OME indications, whereas the US guidelines (R. Rosenfeld, et al. 2004) recommend it together with the second set of VTs. Because many children only have one set of tubes, subjecting all to adenoidectomy will expose many to unjustifiable additional surgical risks, and adenoidectomy itself does not prevent OME recurrence even if all children receive it.

Thus, even with adjuvant adenoidectomy, the outcomes of OME treatment remain unsatisfactory, and 58.7% of those treated with VTs+ad still require further surgery according to this long term study in severely affected children. Any new strategy that may improve the high need for further surgery would be welcome.

5.4.6 How long should any future OME trial last?

Any randomised controlled trial faces the issue of appropriate time scale through which to evaluate a treatment, but in OME the timing issues are particularly challenging, because OME is a variably manifesting, fluctuating and eventually resolving condition. For most types of VTs an initial duration of up to 12 months is reasonable, as it is likely that tubes will have extruded by then. However, any trial needs to be sufficiently long to capture events after
VT extrusion and determine all benefits or risks. On the other hand, having too long a time-frame could be counterproductive, creating practical and resource problems in realising the trial, and setting up an unrealistic scale of lasting and large benefit against which an actual modest and short-term (though possibly worthwhile) result will appear insignificant.

TARGET itself ran for 2 years, but the data presented here indicate that, although much important information can be obtained during 2 years, a significant number of children require care beyond 2 years. Differences beyond 7.5 years from randomisation (the minimum follow-up period in TARGET long term study) are less likely to be of interest, because by that stage children will be at least 11 years old (7.5 years from randomisation + minimum age 3.5 years at randomisation), beyond the age when OME resolves in many of children. However, longer follow up still could add more useful information, particularly as published data suggest that it takes 10 years before OME has resolved in 95% of children (Maw and Bawden 1994) (Rosenfeldt and Bluestone 1999).

The current treatment of OME requires improvement to try and reduce the high recurrence rate. The data presented in subsequent chapters in this thesis suggests that using a modified release antibiotic formulation in the middle ear to treat biofilms associated with OME is a promising future therapeutic strategy. As such, at some stage a clinical trial will be planned to determine the effectiveness of this strategy. So what would be an appropriate time for trial duration? To some extent this is a subjective decision, but 2 years is
clearly not enough, and the trial should also extend beyond the 4.5 years that was the mean duration of care in children receiving VTs. A follow-up time of 7.5 years from randomisation seems a sensible trial duration based on data presented here, although extending this to 10 years would be better still. Following up until a certain age is perhaps a better strategy (as it is known that OME does eventually resolve), and the data here support the notion of follow-up until a child is 14 years old. The trial duration recommendations made here seem sensible on the basis of presented data, but it has to be remembered that if one followed up children longer than in this study, additional visits at a later age could still become apparent.

The duration of future trials may also be in part determined by the outcomes studied, and the ease with which the necessary data can be obtained. If one examines need for repeat surgery then a long term view if necessary, but insisting that all children irrespective of on going problems attend clinic is not. To study hearing, repeat attendances for audiometry would be necessary. If one studies the benefits that adenoidectomy has on nasal obstruction, then a 2 month follow-up period is sufficient, at least initially, because surgery can be expected to have almost immediate benefit. If one studies acute otitis media, follow-up beyond 2 years of age can be limited, because most children grow out of the disease by that age. Medical records follow-up as in TARGET long term study appears to have been a successful, achievable, and affordable way of studying the outcomes of clinic visits and need for further surgery, whilst asking people to return to clinic or fill in questionnaires may have
received little uptake. A future clinical trial could therefore adopt a similar structure as TARGET and its long term follow-up studies.
5.5 TARGET LONG-TERM STUDY: CONCLUSION

The recommended treatment for persistent OME, Ventilation Tube insertion, has significant shortcomings. Examining the long term (>7.5 years from randomisation) outcomes of VTs and VTs+ad, on a subset of children enrolled in the 2-year TARGET randomised controlled study of children with OME lasting at least 3 months and with a hearing loss of at least 20 dB NHL, demonstrates that the clinical care of children having VTs lasts on average 4.25 years from randomisation, repeat surgery is necessary in 63.6% of those allocated VTs, and the average number of procedures per child is 2.27. Although adenoidectomy can reduce the mean number of procedures, the need for repeat surgery remains very high. The research presented here also demonstrates the importance of sufficiently long follow-up of children with OME to ensure that all their clinical care is captured.

Current OME treatment therefore has significant shortcoming, and any new treatments based on thorough understanding of OME aetiopathogenesis could improve the care of countless children. Irrespective of their shortcomings, VTs are currently the only treatment recommended by NICE. But are clinicians actually following these recommendations? The next chapter examines whether children are being listed for surgery in accordance with NICE guidelines, and whether their recent introduction has changed practice.
6 ARE WE FOLLOWING NICE GUIDELINES ON THE
MANAGEMENT OF OTITIS MEDIA WITH EFFUSION?

6.1 INTRODUCTION

Although OME is transient in the majority of children, a proportion develop persistent symptoms that may affect hearing, education, language or behaviour (Hall, Maw and Steer 2009) (NICE 2008). If OME persists after a three month period of watchful waiting, treatment with ventilation tubes (VTs, grommets) or hearing aids may be considered (NICE 2008) (Lous, et al. 2005). With most UK parents opting for surgery, VT insertion is one of the commonest surgical procedures in children (Kubba, Pearson and Birchall 2000), with over 30,000 inserted in England each year (HES 2010).

The UK National Institute of Clinical Excellence (NICE) guidelines on surgical management of OME (NICE 2008) provide detailed guidance on the management of children younger than 12 years with suspected OME, covering history-taking, examination, and audiometric testing, and offering a review of the different management options. The guidelines, applied to “newly diagnosed OME of unknown duration”, call for an initial 3 month period of observation, with repeat audiological assessment at the end of the three month period. At that stage, VT insertion should be considered for children with persistent bilateral OME with a hearing level in better ear of 25–30 dB NHL or worse (termed core criteria in this thesis), or for children not meeting
those audiometric criteria but when OME has significant impact on developmental, social or educational status (termed exceptional circumstances in this text). The guidelines state that surgery is not appropriate for children that have OME only for a short time period or in cases where OME has little effect on child’s function and development. Adenoidectomy is not recommended in the absence of co-present history of frequent upper respiratory tract infection.

We carried out a multi-centre comparison to establish whether children are being listed for surgery in accordance with NICE guidelines, and whether the guidelines have changed clinical practice.
6.2 METHODS

A multi-centre retrospective case-notes review was undertaken, including all children aged 12 years or under having VT insertion before (Jul-Dec 06) and after (Jul-Dec 08) guideline publication in Feb 08. The units involved were Kings Mill Hospital Mansfield (Daniel, L Harrison), Queen's Medical Centre Nottingham (Daniel, Cho), Royal Derby Hospital (Daniel, Kamani, A Harrison), Royal Free Hospital London (Jaberoo, Yalamanchili) and Lincoln County Hospitals (El-Shunnar).

The guidelines were published in February 2008, and the study time period chosen therefore allowed over 4 months for guidelines’ implementation, and all patients included would have been listed for surgery since guidelines publication. To establish whether guidelines had changed clinical practice, a six month period (keeping the time of year the same) prior to the introduction of the guidelines was also studied (1st July to 31st December 2006), applying the same NICE criteria retrospectively.

All children aged less than 12 years having VT insertion were identified using the Hospital Information Support System data, and their medical records reviewed to identify those that had first VT insertion. Children with Down’s syndrome or cleft lip / palate were excluded (NICE guidelines specify a different management pathway), as were those having surgery other than VTs alone or VTs with adenoidectomy, or those with major comorbidities, special
educational needs or other sensory impairment that may affect the decision to list for surgery.

The medical records of the included children were studied to determine whether they were listed for surgery in accordance with NICE guidelines. The following variables were studied:

- Did the child have two audiograms at least three months apart?
- Did the child meet the audiological criteria of a hearing level of 25 dB NHL or worse (averaged at 0.5, 1, 2 and 4 kHz) in the better ear, at time of listing or at the pre-operative audiogram closest to surgery?
- If both above criteria were met the child was deemed to have fulfilled guideline's core criteria.
- Did the child have OME duration of at least 3 months on the basis of history, irrespective of whether the child had two audiograms or not?
- When core criteria were not met, the clinical notes were reviewed to establish whether there was an extenuating reason for surgery, typically concerns over speech or schooling, or recurrent acute otitis media (AOM).

The group of children that either met core criteria or had extenuating circumstances leading to surgery were deemed to have complied guideline's broad criteria.

- What proportion of children complying with the broad criteria achieved this status due to extenuating circumstances?
- Number of children having adenoidectomy was also examined, and we sought to establish whether adenoidectomy was performed purely for OME
or whether there was another reason in the notes that justified adenoidectomy.

Statistical analysis was performed using SPSS Statistics 19. Differences in proportions were analysed using the Pearson Chi squared test or the Fisher's exact test if numbers were small. The Student's t test was used to assess differences in means.
6.3 RESULTS

A total of 319 patients were included in the study. Notes of a further 41 (11.4%) patients could not be obtained despite multiple searches at different times. The five different hospitals contributed 41, 90, 107, 30, and 51 patients, respectively.

Pooled data from all 5 units were used to compare practice in accordance with the set criteria before and after guidelines were introduced. During the pre-guidelines period there were 173 cases, and post-guidelines 146. In three units the number of VTs reduced, and in two increased after guidelines' introduction. The percentage of boys was 61.4% overall with no significant differences between the two time periods, and the mean age during both time periods was 4.7 years.

Table 6.3.1 compares the two time periods. Practice in accordance with the core criteria has deteriorated by a significant amount (Chi squared p=0.032). However, there has been no deterioration in practice in accordance with guidelines if those children listed for extenuating circumstances were included, but this was achieved by a significant rise in the number of such extenuating cases. A variety of extenuating reasons were noted, these are detailed in Fig 6.3.2.
Variation between the five centres was also noted, for example the proportion meeting core criteria after guidelines publication ranged from 21.1% to 39.5%, and the proportion meeting extenuating criteria from 84.2% to 91.3%, but this variation was not statistically significant. Additionally the centre that was least likely to meet core criteria had the most children with recurrent AOM (31.6%) and the centre most likely to meet core criteria had the smallest number of children with AOM (7.9%), so it is likely that variation in AOM rates in the local population may be responsible for at least some variation in listing practice.
<table>
<thead>
<tr>
<th>CRITERION</th>
<th>Pre-guidelines (N=173)</th>
<th>Post-guidelines (N=146)</th>
<th>Change pre- to post-guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 audiograms ≥3 months apart</td>
<td>100/173 (57.8%)</td>
<td>80/146 (54.8%)</td>
<td>↓3.0% (p=0.589)</td>
</tr>
<tr>
<td>Duration ≥3 months</td>
<td>164/173 (94.8%)</td>
<td>135/146 (92.5%)</td>
<td>↓2.3% (p=0.392)</td>
</tr>
<tr>
<td>Hearing 25dB NHL or worse in better ear</td>
<td>118/173 (68.2%)</td>
<td>89/146 (61.0%)</td>
<td>↓7.2% (p=0.177)</td>
</tr>
<tr>
<td>Fulfils core criteria</td>
<td>76/173 (43.9%)</td>
<td>47/146 (32.2%)</td>
<td>↓11.7% (p=0.032)</td>
</tr>
<tr>
<td>Fulfils broad criteria</td>
<td>148/173 (85.5%)</td>
<td>127/146 (87.0%)</td>
<td>↑1.5% (p=0.711)</td>
</tr>
<tr>
<td>Exceptional cases proportion</td>
<td>72/148 (48.6%)</td>
<td>79/127 (62.2%)</td>
<td>↑13.6% (p=0.024)</td>
</tr>
<tr>
<td>Also had Adenoidectomy</td>
<td>37/173 (21.4%)</td>
<td>29/146 (19.9%)</td>
<td>↓1.5% (p=0.738)</td>
</tr>
<tr>
<td>Non-OME reason for adenoidectomy</td>
<td>32/37 (86.5%)</td>
<td>21/29 (72.4%)</td>
<td>↓14.1% (p=0.154)</td>
</tr>
</tbody>
</table>

Table 6.3.1: Practice in accordance with NICE guidelines on OME during the two time periods.
<table>
<thead>
<tr>
<th>REASON FOR EXTENUATING SURGERY</th>
<th>Pre-guidelines (N=72)</th>
<th>Post-guidelines (N=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speech problems</td>
<td>38 (52.8%)</td>
<td>46 (57.5%)</td>
</tr>
<tr>
<td>Schooling problems</td>
<td>27 (37.5%)</td>
<td>25 (31.3%)</td>
</tr>
<tr>
<td>Recurrent AOM</td>
<td>43 (59.7%)</td>
<td>26 (32.5%)</td>
</tr>
</tbody>
</table>

Table 6.3.2: Reasons why children required surgery despite not meeting core criteria.
6.4 DISCUSSION

6.4.1 Summary of findings

Overall, 87.0% of children have VTs inserted in accordance with NICE guidelines in their broadest sense, although this relies on the inclusion of children meeting extenuating circumstances that justified VT insertion despite that child not fulfilling the core criteria. Only 32.2% of children met the core criteria of two audiograms 3 months apart with hearing level at 25dB NHL or worse, and this proportion appears to be lower now than it was prior to guidelines' publication.

6.4.2 Practice in accordance with guidelines

The aim of any guideline, a systematic statement of best evidence-based practice to assist doctors and patients in decision making about appropriate healthcare in specific circumstances (NHS Centre for Reviews and Dissemination 1994), is to inform and standardise best practice. However, whilst guidelines should lead to improved patient care, this is by no means universal (Freemantle, et al. 2000) (NHS Centre for Reviews and Dissemination 1994) (Oxman, et al. 1995). Much OME literature points to great variations in surgery rates and frequent non-compliance with existing guidelines.

In Norway in 2002 the odds of surgery for OME or AOM varied 4 fold across different regions (Karevold, et al. 2007), and up to 10 fold for AOM across different countries (UK having the lowest rate) (Schilder, Lok and Rovers...
2004). Within England, the number of VTs inserted (2007-2010) ranged from 62.1 to 495.1 per 100,000 population (Child and Maternal Health Observatory 2012), which does not seem to be any better than the seven fold variation between different districts reported in the 1980s (N. Black 1985). There was also a 10 fold variation in VT rates within Ontario in Canada in the late 1990s (Coyte, et al. 2001), this variation being larger than that seen in almost any other procedure.

A US study in 2002 found that >90% of VTs were not inserted in accordance with guidelines in place at the time, and 81% were not appropriate according to criteria developed by an expert panel for the study (Keyhani, et al. 2008). Even if children with other conditions that predispose to a poor developmental outcome, those requiring other surgery, or those that had previous VTs were excluded, the number of appropriate VTs was still less than 40%. The number of VTs in the UK was in the past considered excessive, and guidelines published in 1992 (NHS Centre for Reviews and Dissemination 1992) emphasised the fact that for many children OME was a temporary phenomenon and watchful waiting should be the norm for most. The rates of OME surgery in England halved during the 1990s, and although the guidelines undoubtedly contributed, there was already an existing downwards trend in surgery rates (Black and Hutchings 2002). The recent publication of NICE guidelines on the surgical management of OME does not appear to have affected rates of VT surgery in England and Wales (Al-Hussaini, Owens and Tomkinson 2011).
Several possible explanations exist for the variation in surgery rates, ranging from differences in clinical opinion on diagnosis and effectiveness of treatment, to parental preferences, and perhaps financial factors. Of course the problem may not be that surgeons are ignoring the guidelines, it may be that parents and health professionals feel that the guidelines are over-restrictive and do not allow personalisation of care to each individual child. NICE guidelines do make provision for individualised care in those children that do not meet core criteria but in whom extenuating circumstances (such as schooling, speech problems or frequent infections) justify surgery.

6.4.3 Duration of OME

OME is a temporary phenomenon in most children. When discovered on screening of asymptomatic children, it resolves in 63% by three months and in 88% by one year (Rosenfeldt and Bluestone 1999). The NICE guidelines have taken this into account, as treatment is inappropriate for most children with incidentally discovered, short-term, unilateral or asymptomatic OME.

The group of children where surgery may be considered are those with bilateral OME persisting at least three months. This group has a much lower spontaneous resolution rate, only 32% by one year and 49% by three years (Rosenfeldt and Bluestone 1999) (Maw and Bawden 1994), with the median duration of OME being as long as 7.8 years (Maw and Bawden 1993).

Although in this study only just over half of children had two audiograms three months apart, the proportion of children that had OME for three months or
more was in excess of 90%. This apparent discrepancy likely arises through a variety of reasons: in some younger children it may be difficult to obtain two audiograms, further waiting may be inappropriate for someone that has already had hearing loss for a year according to parents, and if a child has already developed speech or schooling problems at first presentation then delays for repeat audiometry may be inappropriate.

6.4.4 Extenuating circumstances justifying surgery

When faced with a child with OME and speech delay, schooling problems, or recurrent infections, it is difficult to justify further delays for repeat audiometry in 3 months, or to not list a child for surgery if audiometric criteria are not met. Provision is therefore made for this in the NICE guidelines. Hence, when these extenuating cases are included with those meeting core criteria, the proportion of children having surgery in accordance with the guidelines in their broad sense rises to 87.0% in the post-NICE period, compared with only 32.2% meeting the core criteria of 2 audiograms three months apart with hearing level of 25 dB NHL or worse in the better ear.

Nevertheless, the high number of children having VTs due to extenuating reasons is surprising. As these cases should, by definition, be exceptional, the observation that they form 62.2% of all children meeting the broad NICE criteria suggests that they are almost the norm, rather than an exception. The proportion of children, amongst those meeting broadest listing criteria, that are having surgery due to extenuating reasons has also risen following guidelines introduction. This may suggest that surgeons are working within the
guidelines, following the suggested listing criteria, and documenting any reasons why surgery was recommended in a child that does not fulfil the core criteria themselves.

6.4.5 Adenoidectomy

As about 20-25% of children having VTs will require re-insertion of tubes within 2 years (Gates, Avery and Prihoda 1987), adenoidectomy as an adjunct to VT insertion has been investigated by several studies (Black, Sanderson, et al. 1990) (Browning 2012) (Casselbrant, Mandel, et al. 2009) (Dempster, Browning and Gatehouse 1993) (Gates, Avery and Prihoda 1987) (Maw and Bawden 1994) (MRC Multicentre Otitis Media Study Group 2001a) (MRC Multicentre Otitis Media Study Group 2001b) (Popova, Varbanova and Popov 2010) (Rosenfeldt and Bluestone 1999). A 1992 review in Effective Health Care concluded that adding adenoidectomy to VTs had a small effect up to 12 months post op (NHS Centre for Reviews and Dissemination 1992), whilst a Cochrane review in 2010 concluded that adenoidectomy has a significantly beneficial effect on OME resolution, although only a small benefit to hearing (van den Aardweg, Schilder, et al. 2010).

In the UK adjuvant adenoidectomy is not recommended in the absence of persistent and/or frequent upper respiratory tract symptoms (NICE 2008). Whilst American guidelines agree with UK stance in the case of primary surgery, they recommend that adenoidectomy is undertaken in conjunction with any revision surgery (R. Rosenfeld, et al. 2004). The fact that most children do not require a second set of VTs is used to argue against
adenoidectomy at the first procedure. There is a great variation in the number of children receiving adenoidectomy in different countries, but on the whole the number of children having adenoidectomy appears to be reducing (Haapkyla, et al. 2008).

This study examined adenoidectomy rates during two time periods, to establish if there has been a reduction in the number having adenoidectomy as a result of the guidelines advising against routine adenoidectomy in new cases of OME. There was no significant difference, suggesting that either most surgeons were either not performing adenoidectomy at the first procedure even before the guidelines, or that they are continuing to perform adenoidectomy despite the guidelines. The number of cases where adenoidectomy was justified for non-OME reasons fell after guidelines introduction (86.5 to 72.4%), but by a non significant amount. Thus, since the guidelines were introduced, in over a quarter of cases this study failed to find justification (within OME guidelines) for adenoidectomy taking place. As this was a retrospective case-notes review, it may well be that adenoidectomy was required, but the reasons why just not documented in the notes. Better documentation could be achieved in a prospective study, but this would not be appropriate when one is examining compliance with guidelines because clinicians' practice would likely be affected by the knowledge that they are being audited.
6.4.6 Limitations

This study is limited by its retrospective nature. The documentation in patient's notes was used for the database, but at the time of the clinic visit there may have been other issues influencing a decision but not being documented. There were also a number of missing case notes, although there are no reasons to suspect that those children would be in any way systematically different.

The study comprised 5 different units, ranging from small district general hospitals to large teaching hospitals. Although the results are representative of the overall practice in the five units studied, individual hospitals should undertake their own audits to assess local practice, as this study may not be representative of other individual units.

The data here analysed only those children receiving surgery, and no comment can therefore be made on practice according to guidelines in those children that were never listed for surgery. Thus, it would be interesting to also examine whether surgery was being denied to children that may in fact have benefited from VTs. It would also be useful to examine if a longer time period for guidelines’ implementation affected the results obtained here, and such a study is planned for the future.
6.5 NICE OME GUIDELINES: CONCLUSION

This study shows that 87.0% of children have VTs inserted in accordance with NICE guidelines provided those children having extenuating reasons for surgery are included. However, only 32.2% meet the core criteria of 2 audiograms three months apart with hearing level of 25 dB NHL or worse in the better ear, a significant number of children are having surgery due to the invoking of the exceptional circumstances criteria, and practice appears not to have changed. Individual hospitals should ensure continued practice within guidelines and improvements by means of regular departmental audit.

This chapter is the final clinical one, as the remainder (and main part) of the thesis explores laboratory aspects of OME, biofilms, and potential new treatments. The clinical chapters, showing that current treatment with VTs carries a high need for revision surgery that is only partially improved by adenoidectomy, and that clinical guidelines on OME management are frequently ignored, provide clear supporting evidence for the need to better elucidate the aetiology of OME and develop improved treatments based on better understanding of OME aetiopathogenesis.
7 BACTERIA IN OME

7.1 INTRODUCTION

Although OME is an extremely common condition, much discussion has taken place regarding its aetiology (Rovers, et al. 2004). OME is a chronic inflammatory condition, with the inflamed mucosa producing mucus that accumulates in the middle ear cleft (Kubba, Pearson and Birchall 2000). However, the cause of this inflammatory response has been controversial.


The investigations of biofilms as causative agents OME has arisen from the PCR / culture discrepancy, because biofilm bacteria may be slow-growing and
difficult to culture in the laboratory, and the localised nature of biofilm communities may make effusion sampling prone to errors and false negative results. Further, the chronic nature of OME, its inflammatory histology in the absence of symptoms and signs of acute infection, and failure of oral antibiotics to confer a lasting benefit would be considered typical of a biofilm infection.

The aim of this section therefore is to investigate the role of bacteria and biofilms in OME. Effusion samples were cultured for a prolonged period of time on a wide range of media, with the intention of capturing all possible bacteria involved in OME. The effusion samples were also analysed using the LiveDead stain with the intention of identifying live bacteria without the need to grow them in the laboratory, and also to determine whether those bacteria existed in biofilm morphology.
7.2 METHODS

7.2.1 Clinical details

The study was approved by Nottingham Research Ethics Committee, and informed consent obtained from participants and parents/carers. Patients were recruited in a large teaching hospital (Nottingham University Queen's Medical Centre), with samples collected between May 2006 and January 2008, and analysed during that time and over the subsequent 2.5 years. Patients were listed for ventilation tube insertion according to standard clinical practice, due to symptomatic OME persisting for at least 3 months. The deliberately wide inclusion criteria were chosen to maximise the applicability of study results to clinical practice.

The ear canal was disinfected by instilling 70% isopropanol for 2 minutes, with external ear canal swabs performed before and after alcohol disinfection. Myringotomy was performed using standard aseptic technique, and effusion aspirated into a sterile collection tube and transported for immediate processing.

7.2.2 Bacterial culture

To identify bacteria in effusion samples, the samples were analysed in a dedicated microbiology laboratory routinely involved in the processing of clinical samples. Effusion samples were cultured on six different media with the intention to capture as many different bacteria as possible. Sheep blood and MacConkey agar plates (Oxoid, Basingstoke, UK) were incubated
aerobically for up to three weeks at 37°C. The remaining four media (Helicobacter pylori, chocolatised blood for Haemophilus influenzae, and Mycoplasma selective agars incubated in 5% CO₂, and sheep blood agar incubated anaerobically), were incubated for up to ten weeks.

7.2.3 Bacterial identification

Bacteria were identified using conventional tests including Gram stain, catalase, oxidase and DNase production, optochin susceptibility, response to growth factors X (hemin) and V (nicotinamide adenine dinucleotide), and growth in anaerobic conditions. Analytical Profile Index (API) strips (bioMérieux, Marcy l'Etoile, France) were used to aid identification and speciate the isolates.

7.2.4 LiveDead stain

The LiveDead stain (Molecular Probes, Oregon, USA) was used according to the product literature protocol, by combining an equal amount of sample with the stain solution, mixing thoroughly, and incubating in the dark prior to examination with appropriate fluorescence filters. The LiveDead stain employs differential membrane permeability to stain intact and uncompromised bacterial cell membranes (i.e. live bacteria) green with SYTO 9, whereas nuclei of dead bacteria stain red with propidium iodide. Eukaryotic-derived material also has a tendency to stain red.
7.2.5 Confocal microscopy

Combining the LiveDead stain with Confocal Laser Scanning Microscopy (CLSM) allows the determination of the three dimensional structure typical of biofilms. CLSM was performed using a Leica SP2 microscope (Leica, Milton Keynes UK), on samples stained with the LiveDead stain. Biofilms were diagnosed on the basis of well-established morphologic criteria (Parsek and Singh 2003) that have been applied to OME in the past (Hall-Stoodley, Hu, et al. 2006), i.e. the presence of three-dimensional bacterial clusters within an amorphous matrix and associated with a surface such as eukaryotic cells or strands.

Two assessors (myself and Roger Bayston) determined bacterial morphology, and both were blinded to the culture result; in case of discrepancies, John Birchall also reviewed the images and a consensus between the three reviewers reached.

7.2.6 Statistical analysis

Statistical analysis was performed using PASW Statistics 18. Differences in proportions were analysed with Chi squared test, or the Fisher's exact test if numbers were small. The Student's t test was used to assess differences in means. Cohen's Kappa coefficient was used to measure agreement between different variables attached to the same sample.

John Birchall and his theatre team collected the clinical samples. Shehla Imtiaz-Umer performed majority of routine microbiology work, with myself
performing the remainder, in addition to collating and analysing data, and performing statistical analysis.
7.3 RESULTS

A total of 62 samples from 42 different patients (27 male, 15 female) were analysed. Most patients (35, 83.3%) were aged under 18 years, the median age being 4.5 years and age ranging from 1 to 75.

7.3.1 Verification of canal disinfection

A random subset of 14 ears was analysed to assess effectiveness of alcohol disinfection, by comparing culture results in external ear canal swabs taken before and after disinfection. Four swabs were culture positive prior to disinfection (one containing *Streptococcus constelatus*, one *Bacillus* spp, and two coagulase negative staphylococci). All swab cultures were negative after disinfection.

7.3.2 Effusion culture and confocal microscopy

Twenty-eight of the 62 effusions were culture-positive (45.2%), but CLSM demonstrated live bacteria in 51 (82.3%) samples. Table 7.3.2.1 illustrates the relationship between culture and CLSM results, showing that only 8.1% of samples were negative on both examinations. In other words, combining the two techniques identified the presence of live bacteria in 57 effusions or 91.9%.
Table 7.3.2.1: Comparison of culture and confocal microscopy results; percentages refer to the per cent out of all 62 samples.

<table>
<thead>
<tr>
<th></th>
<th>CLSM-positive</th>
<th>CLSM-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-positive</td>
<td>22 (35.5%)</td>
<td>6 (9.7%)</td>
<td>28 (45.2%)</td>
</tr>
<tr>
<td>Culture-negative</td>
<td>29 (85.3%)</td>
<td>5 (8.1%)</td>
<td>34 (54.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>51 (82.3%)</td>
<td>11 (17.7%)</td>
<td>62</td>
</tr>
</tbody>
</table>

Among the CLSM-positive samples, 25 (49.0%) contained biofilms and 26 (51.0%) planktonic bacteria. Culture positivity appeared not to influence bacterial morphology at CLSM, as the proportion of samples exhibiting biofilm morphology was similar in the culture-positive and culture-negative groups (11/22, 50.0% and 14/29, 48.3%, respectively).

Differences between adults and children were explored on a per-patient (rather than per-ear) basis; where data existed for two ears, the per-patient analysis was carried out using the criteria of at least one ear being culture / confocal positive and at least one ear containing biofilms. Children appeared to have a greater number of culture-positive, confocal-positive, and biofilm results than adults (54.3 v 14.3%, 82.9 versus 57.1%, and 67.9 v 0%, respectively). However, only the presence of biofilms reached statistical significance (Fisher’s exact test p=0.02).

In the 20 patients (all children) in whom data from both ears were available the correlation between the findings in the two ears was assessed. The agreement rate was 70.0% (14/20) in the case of culture positivity rate (kappa=0.381, fair agreement), 90.0% (18/20) in the case of confocal positivity
rate (kappa=0.444, moderate agreement), and 58.8% (10/17 confocal positive samples) in the case of confocal morphology type (kappa=0.168, poor agreement). The same bacteria were identified in both ears in 7 children (35.0%)

Table 7.3.2.2 shows the range of different bacterial isolates, including the relationship of bacterial isolates to CLSM findings. The mean number of different bacterial isolates was 1.8 per culture-positive sample, with no significant difference between confocal-positive and confocal-negative samples. Biofilm samples contained an average of 1.7 and planktonic samples 2.0 different bacterial isolates, but this was not statistically significant on t-test (p=0.51). Only one adult patient’s effusion was culture positive, growing S capitis and Acinetobacter radioresistens, with most bacteria identified thus coming from children’s effusions.

Figure 7.3.2.1 shows representative images obtained with CLSM.
<table>
<thead>
<tr>
<th>BACTERIA</th>
<th>N (%) isolates among all 62 samples</th>
<th>Confocal microscopy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CoNS</td>
<td>8 (12.9)</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Veillonella spp</td>
<td>6 (9.7)</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5 (8.1)</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Strept pneumoniae</td>
<td>4 (6.5)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus spp</td>
<td>3 (4.8)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>3 (4.8)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas spp</td>
<td>3 (4.8)</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter spp</td>
<td>2 (3.2)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corynebacter propinquum</td>
<td>2 (3.2)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Flavimonas oryzihabitans</td>
<td>2 (3.2)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>2 (3.2)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>2 (3.2)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vibrio metchnikovi</td>
<td>2 (3.2)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gemella haemolyans</td>
<td>1 (1.6)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kocuria varians / rosea</td>
<td>1 (1.6)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcus sp</td>
<td>1 (1.6)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptococcus sp</td>
<td>1 (1.6)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3.2.2: Different bacterial species isolated from effusion samples, and their relationship to confocal microscopy findings. Number (N) refers to number of isolates rather than number of samples. The coagulase-negative staphylococci (CoNS) isolates were 2 *S. lugdunensis*, 2 *S. epidermidis*, one each *S. simulans*, *S. capitis* and *S. hominis*, and one sample contained both *S. capitis* and *S. lugdunensis*. Acinetobacter consisted of one *A. lwofii* and one *A. radioresistens*, and *Pseudomonas* consisted of one *P. aeruginosa*, one
*P. stutzeri* and one *P. luteola* isolates. Majority of bacteria were isolated from children's effusions, with only one adult patient's effusion being culture positive (containing *S. capitis* and *A. radioresistens*).

Figure 7.3.2.1 (following pages)

CLSM images showing live bacteria staining bright green, whereas dead bacteria and eukaryotic cells/material/DNA stain red. A: Copious live bacteria staining green, some as biofilm clusters associated with orange-staining eukaryotic material (arrows), and some planktonic as single free bacteria (circled). B: Three dimensional representation of bacterial clusters (arrows) associated with red-staining eukaryotic material, plus planktonic bacteria (circled). C: Copious strands with biofilms (arrows) and planktonic bacteria (circled), with the same section represented three-dimensionally in D. E: Strands associated with biofilms plus planktonic bacteria, with the same section represented three-dimensionally in F. G: Clump of live bacteria staining bright green and some dead bacteria staining red, surrounded by an amorphous matrix staining dull green. H: Three-dimensional representation of bacteria (bright green) in an amorphous matrix (dull green) attached to red-staining eukaryotic material.
7.4 DISCUSSION

7.4.1 Summary of findings

The results show that combining culture and confocal microscopy demonstrates live bacteria in more than 90% of OME samples, strongly suggesting a role for bacteria in the aetiopathogenesis of OME. Extended cultures alone were positive in 45.2%. However, CLSM was positive in 82.3%, and in these samples biofilms were demonstrated in 49.0%. A wide variety of different bacteria were also identified, rather than just the traditional three studied in the past (Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis).

7.4.2 Biofilms in OME

The microbiology of OME has been studied for many decades, yet controversy still exists as to the role of bacteria in the aetiology of OME (Kubba, Pearson and Birchall 2000) (Parkinson, et al. 2006). A high proportion of effusions (typically more than half) are culture-negative (Matar, et al. 1998) (Gok, et al. 2001) (Schousboe, et al. 2001) (Poetker, et al. 2005) (Bluestone, Stephenson and Martin 1992). However, while PCR has demonstrated bacterial nucleic acids in a much greater number of patients (Hendolin, et al. 1997) (Post, et al. 1995), nucleic acids identified at PCR may not represent viable bacteria. An alternative view of bacteria in OME is that they might be viable but not culturable by conventional means, a state that is commonly seen in biofilms (Proctor and von Humboldt 1998) (Proctor, et al. 2006).
Using CLSM we were able to detect biofilms in just under half of CLSM-positive samples. However, CLSM identified live bacteria in 85.3% of the culture-negative samples. Among culture-negative samples 14.7% were also confocal-negative, but of the culture-positive samples 21.4% were confocal-negative. This suggests that CLSM may under-estimate the presence of bacteria, perhaps due to sampling differences, as small samples of effusion must be further divided for each examination and bacteria may be very localised. There appeared to be no great difference between planktonic and biofilm bacteria identified on LCMS-positive samples, with similar numbers being culture-positive. Although one might expect all planktonic samples to be culture-positive, 16 planktonic effusions were culture-negative. It is possible that these bacteria did exist as biofilms (which may make them difficult to culture), but that original morphology was disturbed by the process of surgical aspiration or laboratory preparation/mixing. The fact that some effusions containing biofilms on confocal microscopy were culture positive is not surprising as bacteria from biofilms frequently leave the biofilm to enter the planktonic state (and would therefore be culturable), and processing of samples for analysis may also disrupt the biofilm thus rendering the bacteria planktonic and then culture positive.

Adults were included in this study to boost patient numbers. However, children's effusions appear to be more likely to contain biofilms than adults, in keeping with the expected infectious aetiology of OME in children but possibly a different aetiology in adults (Kubba, Pearson and Birchall 2000). In addition,
only one adult effusion was culture positive, again supporting the notion that paediatric OME aetiology is likely to be different to adult OME.

No great correlation between findings in the opposite ears has been demonstrated, suggesting that individual ears may be subject to different local aetiopathogenic mechanisms. This is also in keeping with clinical findings that the two ears in a child often behave differently, and it is not uncommon to have unilateral OME.

The question of whether OME is a biofilm infection has been addressed by Fergie et al (Fergie, et al. 2004). Parsek and Singh suggested that the following criteria should be fulfilled for a disease to be considered a biofilm infection (Parsek and Singh 2003):

- Bacteria are adherent to substratum or a surface
- Bacteria are living in cell clusters or micro-colonies encased in an extracellular matrix
- Infection is generally confined to a particular location.
- Infection is difficult or impossible to eradicate with antibiotics, despite the responsible bacteria being susceptible to the antibiotic when in the planktonic state.

This study clearly demonstrated that the bacteria were alive (something that PCR or DNA-based methods cannot do) in more than 90% of samples, biofilms were found in just under half of effusions. However, studies of middle ear mucosa (not effusions) have shown biofilms more frequently, perhaps in
65 - 92% of samples (Thornton, et al. 2011) (Hall-Stoodley, Hu, et al. 2006), whilst non-invasive *in vivo* optical techniques have demonstrated biofilms in all adults with OME but in none of the controls (Nguyen, Jung, et al. 2012). The seemingly higher biofilm presence in published literature does not necessarily contradict our findings as there are several reasons why a difference between mucosa and mucus may exist. It is possible that biofilms exist on the mucosa in most people with OME but exist within the effusion associated with mucins in only just under half of cases, or that biofilms on mucosa act as reservoirs of bacteria that then enter the effusion, or that there is an issue with which part of the effusion is sampled for analysis. The presence of an intact mucus layer appears to be important in controlling bacteria within the human gastrointestinal tract, as most bacteria reside on the luminal side of the mucus layer (van der Waaij, et al. 2005), with no direct contact with the epithelium unless inflammation is present (Swidsinski, et al. 2007) (Macfarlane and Dillon 2007). To the best of our knowledge we were the first group to assess middle ear effusions with LiveDead staining and confocal microscopy. A paper has been published since our work, but this analysed 4 children's effusions finding no biofilms (Homøe, et al. 2009); with such a small number it is difficult to draw conclusions, especially as according to our data only half effusions contain biofilms. Another recent paper used fluorescence *in-situ* hybridisation, with species-specific probes, to look for biofilms in the effusion of children with OME of less than 3 months' duration, finding biofilms in 4 of 13 (30.8%) effusions (Chen, et al. 2012); effusions were centrifuged prior to analysis, which may have affected biofilm morphology.
But could biofilms be a part of the normal middle ear flora rather than the pathogenic cause of OME? While two studies did not find biofilms in mucosal biopsies of their healthy controls (Hall-Stoodley, Hu, et al. 2006) (Thornton, et al. 2011), another study (Tonnaer, et al. 2009) of middle ear mucosal biopsy at time of cochlear implant showed biofilms in 2 out of 45 cases without evidence of previous otologic problems or abnormalities, suggesting that biofilms may exist in the asymptomatic middle ear. The difficulty when researching biofilms in OME is choice of control group. Entirely normal patients do not require ear surgery, and all the control groups chosen are patients having surgery for another (ostensibly non-related) reason, but they cannot be considered to be normal. When studying effusions as in this study, the problem is compounded by the fact that the normal ear does not have an effusion, therefore no suitable negative control exists. An alternative might have been to study the mucus produced by normal middle ear mucosa, but this is produced in only minute quantities. It would be difficult to sample with anything other than a swab, and whilst culture would be possible, confocal microscopy of strands of actual mucus would not. Further, it also raises the ethical issues of taking samples from healthy people, as surgical access to the middle ear would be required. Even if one included patients having surgery for non-OME reasons, it is still an additional procedure not related to the treatment of that (control) patient, and the assumption that the patient having middle ear surgery is truly healthy and free from OME may be false. A further problem relates to the common phenomenon of temporary OME, so it possible that children considered "normal" may have biofilms in their middle ear.
because they have a temporary problem that would never come to medical attention otherwise.

Despite difficulty studying biofilms in OME, it is recognised that the presence of a biofilm (bacteria or bacterial products such as lipopolysaccharide) may lead to an inflammatory stimulus (Bemthal, et al. 2010) (Mittal, et al. 2009) (Foreman, Jervis-Bardy and Wormwald 2011), which may then result in middle ear mucosal inflammation via cytokine release, mucous cell hyperplasia, and ultimately clinical OME (Lin, et al. 2012) (Smirnova, et al. 2002) (Schousboe, et al. 2001) (Stamer, et al. 2006). In chronic rhinosinusitis (Foreman, Jervis-Bardy and Wormwald 2011), another mucosal biofilm condition, the presence of biofilms was shown to be associated with alteration in host immune responses including those involving interferon gamma, granulocyte-colony stimulating factor, and macrophage inflammatory protein 1-beta, with a local (not systemic) inflammatory response (Hekiert, et al. 2009); biofilm presence has also been associated with marked destruction of the epithelial layer with complete absence of cilia, whereas those patients without biofilms either had normal mucosa or only partial mucosal damage (Galli, et al. 2008).

It is also possible that different biofilms, perhaps at different times, in different positions, or when affected by different immune systems, lead to different pathological problems, in some cases contributing to disease and in others not; in the same way that different bacteria may give different diseases, different biofilms may give different pathology (Foreman, Jervis-Bardy and
Biofilms may also act as a reservoir of bacteria, with some bacteria leaving the biofilm to become planktonic and disseminate. The observation of biofilms on ventilation tubes of children with chronic ear discharge suggests that the middle ear bacterial biofilms have at least the potential to be pathological (Barakate, et al. 2007), whilst the reduction in post-ventilation tube surgery ear discharge achieved through application of antibiotic drops at the time of surgery suggests that treatment directed against biofilms in middle ear disease may be possible (Thomas, Ojano-Dirain and Antonelli 2011) (Shinkwin, et al. 1996).

7.4.3 Techniques and alternatives

Biofilms were diagnosed on the basis of well-established morphologic criteria (Parsek and Singh 2003) that have been applied to OME in the past (Hall-Stoodley, Hu, et al. 2006): three-dimensional bacterial clusters within an amorphous matrix and associated with a surface such as eukaryotic cells or mucus strands were classified as biofilms. In contrast, bacteria that appeared as individual bacteria rather than a grouping were considered planktonic, as were any bacterial groups that were not associated with a surface. Typical examples are shown in Fig 7.3.2.1.

Although the BacLight stain enables differentiation only between bacteria with intact and damaged cytoplasmic membranes it is often used to differentiate between active and dead cells (Sachidanandham, Gin and Poh 2005) (O'Neill, et al. 2004), and while it appears to be accurate to consider membrane-compromised bacterial cells as dead, the reverse (i.e. intact membrane
signifies live cells) may not be true in a small number of cases (Berney, et al. 2007). However, given that 82.3% of the samples in this study stained live on confocal microscopy, a small false-live rate will still leave the majority of samples as containing live bacteria. Combining the LiveDead stain with confocal microscopy was felt to be the best way of identifying any live but non-culturable bacteria, and of determining the bacterial morphology to establish whether biofilms were present.

PCR has been used in the past (Kubba, Pearson and Birchall 2000) (Hendolin, et al. 1997) (Post, et al. 1995) (Rayner, et al. 1998), but it cannot differentiate between DNA from live and dead bacteria, even when the quantitative technique of real time PCR is used (Saukkoriipi, et al. 2002). Interestingly, a novel technique of differentiating whether DNA comes from live or dead bacteria has recently been developed (Kobayashi, et al. 2009). This uses the DNA-binding agent propidium monoazide to help identify DNA from live bacteria at PCR, and could be usefully applied to OME.

LiveDead staining and confocal microscopy does not identify bacterial species. Therefore, Hall-Stoodley et al (Hall-Stoodley, Hu, et al. 2006) in their study on mucosal biopsies used Fluorescence In-Situ Hybridisation (FISH) to identify bacterial species. However, FISH identifies only those bacteria that are looked for with a species-specific probe, and would miss any that are not specifically sought (unless one uses a pan-bacterial probe, but the identification then again becomes an issue). The adoption of a broad microbiological approach in this work, i.e. to aim to find any bacteria, rather
than designing experiments to find S pneumoniae, H influenzae and M catarrhalis because that is what everyone else has found, is in fact a strength of this work. In relation to FISH, one could find only those bacteria that one looked for, and as our culture results identified 17 different bacteria that would mean running 17 different FISH experiments, and one would still miss identification of bacteria that we found in samples that were positive only by confocal microscopy and negative on culture. Interestingly, recent technological advances have combined PCR with mass spectrometry into automated technology that can detect a wide range of pathogens (Ibis T5000 Universal Biosensor); although based on PCR with its associated limitations, this could be a useful technique in identification of bacteria in OME (Ecker, Drader and Gutierrez 2006). The future may also bring instruments that could visualise bacterial biofilms in situ in the middle ear, as new optical methods capable of detecting them are already being developed (Nguyen, et al. 2010) (Nguyen, Jung, et al. 2012).

SEM has also often been used to identify biofilm. However, whilst biofilm identification on inert surfaces is usually straightforward, its usefulness on mucosal surfaces remains debatable due to difficulty differentiating biofilms from mucus. Therefore, confocal microscopy combined with the non-specific LiveDead stain or species-specific FISH is currently thought to be the gold standard method of identification of biofilms on mucosal surfaces (Foreman, Jervis-Bardy and Wormwald 2011).
7.4.4 Bacterial types in OME

*S. pneumoniae, M. catarrhalis and H. influenzae* are the most common pathogens implicated in OME, and all are capable of forming biofilms (Starner, et al. 2006) (Hall-Stoodley and Stoodley 2009). However, rather than focusing just on those three bacteria, this study cultured effusions on a wide range of different media for prolonged time periods in order to capture as many isolates as possible. Interestingly, coagulase negative staphylococci (CoNS), *Veillonella spp* and *S. aureus* were the three commonest pathogens isolated in this study. CoNS were long thought to be non-pathogenic commensals, but with the recognition of their biofilm-forming capacity have emerged as the leading cause of biomaterials-related infection (McCann, Gilmore and Gorman 2008) (Lyte, et al. 2003). They have also been previously implicated in otitis media (Bunse, et al. 1987), with one study finding CoNS in a third of effusions and a third of these isolates were of the slime-producing phenotype (Paluch-Oleśa, et al. 2011). *Veillonella* is a Gram-negative anaerobe that inhabits the mouth and upper respiratory tract, forms biofilms (Palmer, Diaz and Kolenbrander 2006) and has previously been found in middle ear disease (Brook 1996). *S. aureus* also forms biofilms (Otto 2008) and has been identified in middle ear disease (Hyden, Akerlind and Peebo 2006). Although most of the bacteria in Table 2 have previously been isolated in middle ear disease, to the best of our knowledge *Flavimonas oryzihabitans, Vibrio metschnikovii* and *Gemella haemolysans* have not been implicated previously.

This study found two effusions containing *Helicobacter pylori*. Some studies have suggested that *H pylori* is implicated in the aetiology of OME (Yilmaz, et
al. 2006), although others have not confirmed this (Ozcan, et al. 2009). Interestingly, bile acids and pepsin have also been found in the middle ear (Klokkenburg, et al. 2009) (He, et al. 2007), but whether they are important in the pathogenesis of OME is not clear.

It is unclear why the present study identified the three traditional OME bacteria in a lower proportion of samples than other studies. Variation in prevalence of OME over time has been previously documented (Bluestone, Stephenson and Martin 1992), and may be due to patterns of antibiotic use or vaccination, particularly with the introduction of vaccines against *H. influenzae* type *b* and *S. pneumoniae*. The adoption of a broad microbiological approach in this study with the aim of identification of all bacteria may also have detected species missed by studies adopting a narrower culturing approach.

Although numbers of isolates of any one bacterial species are small, it is interesting to note that majority of CoNS and *S. aureus* appeared in planktonic form, despite both being recognised as biofilm-forming pathogens (McCann, Gilmore and Gorman 2008) (Lyte, et al. 2003) (Otto 2008). *Pseudomonas aeruginosa*, which is well known to produce biofilms (Hall-Stoodley and Stoodley 2009), was also identified in biofilm morphology in this study, but *Acinetobacter* appeared difficult to identify on CLSM.

### 7.4.5 OME biofilms in relation to OME natural resolution

If biofilms cause OME, then why does OME resolve spontaneously in most children by the age of 10 years or so? And if biofilms cause OME, it may be
easy to understand why OME often recurs after VTs have extruded (VTs only masked the effusion but didn’t treat the underlying biofilm), but why then does OME not always recur after VT extrusion? Why is every AOM episode not followed by OME?

The last question is perhaps easiest to answer, as establishment of a biofilm will depend on the pathogenicity of the bacteria involved, and their interaction with the host immune response (Mittal, et al. 2009). The host response may also be the reason why biofilm infections resolve. Younger children have more infections, perhaps due to relatively immature immune system, but perhaps also due to higher exposure to bacteria in communal areas such as school or nurseries, or exposure to bacteria encountered for the first time. However, the major immune maturations occur during the first two years of life (West 2002), hence it is difficult to see how immune maturation would cure glue ear in 3-7 year olds. Environmental and behavioural changes can explain reduced infections in older children, but not the eradication of an established biofilm.

Changes in the Eustachian tube may explain resolution of OME and biofilms. In children, the Eustachian tube is shorter, with the muscular attachments that normally open it being less favourable (Doyle and Swarts 2010). Hence, the mature tube will be less likely to allow reflux of nasopharyngeal contents into the middle ear, preventing infection in the first place, but the tube in an older child will also be opening and aerating the middle ear better.
The aeration of the middle ear may lead to drying of the biofilm and therefore its eradication. Certainly drying may be important as an adjunct to biofilm eradication for example following disinfection of a surface (Kovaleva, Degener and van der Mei 2010), but it appears not to be effective on its own (Wu, et al. 2010) (Mordhorst, et al. 2009). However, aeration and drying may also have an effect on the middle ear mucosa itself. In the presence of inflammation, deficient ventilation, and high CO2, the middle ear mucosa metaplasies into numerous mucus-producing cells (Sade and Weissman 1977); the reverse may also be true, so that restoration of normal ventilation (either with VTs or by age-related improvements in Eustachian tube function) could reduce the number of mucus-producing cells and therefore lead to resolution of effusion.

When discussing OME as a biofilm infection, the question that then arises is whether it really is the biofilms that caused the inflammation leading to the effusion in the first place, or did the effusion develop as a result of middle ear hypoventilation and the biofilms simply moved into the abundant mucus? It has already been stated in the introduction that OME cannot be caused simply by Eustachian obstruction (the effusion would then be a transudate, not an exudate), and the fact that effusions drain down the Eustachian tube almost immediately after VT insertion again suggests that the Eustachian tube was never blocked (Kubba, Pearson and Birchall 2000). Therefore, it seems unlikely that negative pressure as a result of Eustachian blockage is the initiating event; rather, the biofilms are likely to be responsible for the initial inflammatory insult leading to OME. Of course it may be possible that biofilms develop only in the presence of middle ear hypoventilation, or conversely that
Eustachian tube dysfunction arises because of the biofilm itself. It is interesting to note that in the case of the gastritis-causing *Helicobacter pylori* the presence of mucin actually inhibits biofilm production (Cole, et al. 2004), and in the case of lactobacilli the type of mucus affects the degree of adhesion (Li, et al. 2008).

Although it seems likely that biofilms are responsible for initiating OME, improvements in aeration with age or VTs may be responsible for OME resolution. Hypoxia is known to have a potent pro-inflammatory effect on the respiratory epithelium (Steinke, Woodard and Borish 2008), whilst healthy middle ear mucosa permits bidirectional gas exchange between capillaries and the middle ear cavity (Ikarashi, Takahashi and Yamamoto 1999). It may be that better aeration improves the “health” of the mucosa and tips the balance away from biofilms and in favour of production of a smaller amount of less viscous mucous, which in turn may lead to biofilm clearance.

### 7.4.6 Implications for glue ear management

This study clearly implicates bacteria in the aetiopathogenesis of OME. Biofilms are likely to be important, as our study found them in 49.0% of confocal-positive effusions, and previous research has demonstrated them on more than 90% of mucosal biopsies in children with OME or recurrent AOM (Hall-Stoodley, Hu, et al. 2006). This new understanding of OME aetiology also leads to potential novel therapeutic possibilities that may improve the current management options.
At present, treatment of persistent symptomatic OME involves drainage of the effusion and VT insertion, but this does not address any persistent bacterial infection, and serves to merely remove the effusion that is the result of a middle ear inflammation. If the biofilm hypothesis is true, it is perhaps not surprising that more than a quarter of cases will require further surgical treatment (Chapter 5) (Gates, Avery and Prihoda 1987). A good way of confirming this would be to study microbiology of OME in children both at their first and also any subsequent treatment, for if biofilms persist then one may expect to find the same bacteria at second treatment. A literature search failed to find any papers comparing bacteriology at first and subsequent VT insertions, and at any rate the bacteria may be different simply because different bacteria have been acquired and replaced the original biofilm, or the same bacteria may be found at the second time not because they persisted in the middle ear but simply because they re-ascended from a colonised adenoid.
7.5 BACTERIA AND BIOFILMS IN OME: CONCLUSION

The role of bacteria in OME has been controversial, with a long-held debate fuelled by the low number of effusions that are culture-positive and the controversy over whether PCR-positive samples indicate live bacteria. However, this study, using extended cultures and confocal microscopy, demonstrates live bacteria in more than 90% of middle ear effusions in children with glue ear, strongly suggesting that bacteria and biofilms are important in the pathogenesis of the condition.

This understanding also opens avenues for potential new, better treatments for OME. Unlike planktonic bacteria, which would be expected to respond to conventional antibiotic treatment, bacteria in biofilms adopt a distinct phenotype with a slow growth rate that makes them recalcitrant to standard antibacterial therapy (Lewis 2001) (Hall-Stoodley and Stoodley 2009) (Sihorkar and Vyas 2001). Delivering high dose antibiotics directly to the middle ear is an interesting new strategy, and this thesis therefore moves on to look at biofilm eradication in the context of OME biofilms.
8 AN IN VITRO BIOFILM MODEL

8.1 INTRODUCTION

The observation that biofilms are implicated in the aetiology of OME opens an exciting avenue for potential novel treatments. As at present about 25% of children require grommet (ventilation tube, VT) insertion for a second time (Gates, Avery and Prihoda 1987) (Maw and Bawden 1994), and VTs themselves have been associated with problems such as tympanic membrane perforation and scarring (Daly, et al. 2003) (Le, Freeman and Fireman 1991) (Brown, Richards and Ambegaokar 1978) (Bonding and Tos 1985) (Maw and Bawden 1994), better treatment based on new understanding of the aetiology of OME is clearly required. Treatments aimed specifically at biofilms were therefore explored.

We set out to develop an in vitro biofilm model that could be used to study biofilms and treatments directed against them. The test organism chosen for these experiments was Staphylococcus aureus. S aureus is a typical biofilm-forming organism, it is robust, grows well as a biofilm, and is difficult to eradicate (Ojano-Dirain and Antonelli 2012). Although other bacteria (S pneumoniae, M catarrhalis and H influenzae) may be more frequently encountered in OME in published literature, they may also be more fragile in the laboratory setting than S aureus, and thus their absence at the end of an experiment may simply be a reflection of their fragility rather than an indication
of treatment success. Thus, *S. aureus* was deemed a suitable starting point for investigating biofilm eradication with antibiotics.

Whilst several *in vitro* biofilm models are available, an important consideration in our work on OME was to use a model with dimensions that are similar to the middle ear. Ultimately, we planned to test an antibiotic pellet for use in the middle ear, and thus the *in vitro* model would have to have dimensions that are comparable to the human middle ear.
8.2 METHODS

The *S. aureus* biofilms were established as detailed below. To use the models described as a model of biofilms, both physical and functional characteristics of biofilm need to be shown, with demonstration of reduced susceptibility to antibiotics essential (Donlan and Costerton 2002). Therefore the silicone discs were imaged using Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM), and the sensitivity to antibiotics of biofilm bacteria was compared with planktonic bacteria.

To assess effect of treatment on biofilms grown on discs, the biofilms had to be removed from the substratum, and the bacteria re-suspended prior to plating onto agar. In order to ensure that only biofilm bacteria are counted, and not non-attached planktonic bacteria, the discs had to be rinsed carefully on removal. The model was therefore investigated with respect to the number of rinses required to remove non-attached bacteria, duration of sonication required to remove attached bacteria and whether sonication affected bacterial viability, effect of trypsin on bacterial detachment, and whether antibiotic solutions added to the models were effective for the duration of experiment.

8.2.1 An *in vitro* biofilm model

To study biofilm eradication an *in vitro* model was required, and set up as follows. Overnight cultures of *Staphylococcus aureus* (laboratory strains derived from OME) were sampled with an inoculation loop and suspended in
Tryptone Soya Broth (TSB) (Fig 8.2.1.1 and 8.2.1.2), to an optical density of 0.08-0.13 (McFarland 0.5 equivalent) at 490nm measured using a Jenway Multicell Changer Spectrophotometer (Bibby Scientific Limited, Stone UK). The rifampicin and clindamycin MICs of the inoculum were established using the Etest strip (AB Biodisk, Solna, Sweden) on Iso-Sensitest agar (Oxoid, Cambridge UK). Silicone discs (Silicone elastomer, Goodfellow, Cambridge UK, 6.0mm diameter and 1.0mm thick) were placed into the solution, and incubated at 37°C, initially on an orbital shaker (Stuart orbital incubator S150, Jencons-PLS, Lutterworth UK) for one hour and then static for one hour. The discs were rinsed three times in PBS prior to use. Clean control discs were prepared in an identical manner except that the TSB was not inoculated with bacteria (these controls were used so that any contamination of the models can be identified easily). The discs were then aseptically placed into a sterile bijoux bottle containing 2 mL TSB. The bijoux bottles were incubated at 37°C (Firlabo Incubator HPE, Firlabo, Meyzieu France). Planktonic bacteria were also investigated, by inoculating 20 μL of bacterial suspension into the TSB in a bijoux bottle.
Silicone discs (6mm diameter, 1mm thick) from OME cultures.

**TSB**

Minimum Inhibitory Concentration test

37°C
1hr rotatory shaker
1hr still

Tryptone Soya Broth

Non-inoculated clean control discs

**Fig 8.2.1.1:** Establishing biofilms for *in vitro* experiments

**Fig 8.2.1.2:** Silicone disc in TSB in a bijoux bottle.
8.2.2 Scanning electron microscopy

For scanning electron microscopy (SEM), bacterial groupings were fixed in cold acetone for 1 hour and air-dried from tetramethylsilane, sputter coated with gold for 4 minutes, and examined with Jeol 6060LV variable pressure SEM (Jeol UK Ltd, Welwyn Garden City UK). Coating with a heavy metal ensures that the surface of the disc is electro conductive and does not accumulate electrostatic charge when bombarded by electrons, and it also enhances signal and surface resolution.

As SEM requires sample dehydration (the SEM chamber is a vacuum as any gas would spread and attenuate the electron beam), it may result in significant distortion of the biofilm structure, and therefore some biofilm discs were also examined with Environmental Scanning Electron Microscopy (ESEM) which allows examination of whole hydrated biofilms, although with reduced resolution and visualisation of individual bacteria. Silicone discs were examined with Phillips XL30 ESEM-FED (FEI, Eindhoven Netherlands).

8.2.3 Alcian blue stain

The presence of extracellular matrix was examined using Alcian Blue (AB) stain. Biofilms were stained in situ on Phosphate buffered saline-rinsed discs, using Alcian Blue kit (HD Supplies, Aylesbury, UK) for 30 minutes, rinsed and examined using light microscopy (Nikon Eclipse TE200, Nikon Instruments Europe, Kingston UK).
8.2.4 Confocal microscopy

Combining the LiveDead stain with Confocal Laser Scanning Microscopy (CLSM) allows the determination of the three dimensional structure typical of biofilms. CLSM was performed using a Leica SP2 microscope (Leica, Milton Keynes UK), on samples stained with the LiveDead stain. The LiveDead stain (Molecular Probes, Oregon, USA) was used according to the product literature protocol, by combining an equal amount of sample with the stain solution, mixing, and incubating in the dark prior to examination with appropriate fluorescence filters.

The usefulness of the Live/Dead stain was also examined, by comparing results from the Live/Dead stain with culture results. As they both measure live bacteria, the results of the two tests may be expected to be very similar. A comparison between LiveDead stain and culture results was therefore undertaken, using pooled data from biofilm discs that were analysed during the study of the eradication of 1 and 5 day old S aureus (F2318) biofilms with antibiotic solution. Cohen's Kappa coefficient was used to measure agreement between different variables attached to the same biofilm grown in vitro, analysed using PASW Statistics 18.
8.3 RESULTS

8.3.1 Demonstrating biofilm structure

The biofilms were grown on roughened silicone discs (Fig 8.3.1.1), as the rough surface provides easier attachment for biofilms than a smooth surface and therefore represent an environment favourable to biofilm formation (Almaguer-Flores, Ximénez-Fyvie and Rodil 2010). Although the presence of bacteria was often noted by the presence of a cloudy-appearing TSB (Fig 8.3.1.2), the presence of biofilms on the discs was examined using SEM, Environmental SEM (ESEM), confocal microscopy and Alcian blue polysaccharide staining.

Fig 8.3.1.1: Light microscopy of the silicone disc (x1000)
Fig 8.3.1.2: Cloudy appearance of the TSB indicating presence of bacteria (B), compared with an un-inoculated disc (C).

The images from 1 day old biofilms of Staph aureus F2387 examined with SEM and ESEM, and from 5 day old biofilms examined using SEM, are shown in Figures 8.3.1.3 and 8.3.1.4, respectively. Images of SEM from 5 day old biofilms of another S aureus strain, F2315, are shown in Fig 8.3.1.5.
Fig 8.3.1.3: One day old bacterial biofilms (*S. aureus* F2387) seen with SEM (A-D; Jeol 6060LV variable pressure SEM) and with ESEM (E, F; Phillips XL30 ESEM-FED).
Fig 8.3.1.4: Five day old bacterial biofilms (S. aureus F2387) seen with Jeol 6060LV variable pressure SEM (B to F). A: no bacteria control.
Fig 8.3.1.5: Bacterial biofilm (S. aureus F2315) on day 5 seen with Jeol 6060LV variable pressure SEM.
LCSM with LiveDead stain was also used to demonstrate the three-dimensional nature of bacterial biofilms on silicone discs. Biofilms were either examined in situ on the disc, fixed on the disc, or the biofilm was scraped off the silicone disc and examined separately on a slide (Fig 8.3.1.6).

Fig 8.3.1.6 (see images on following pages): LCSM showing S aureus (F2387) biofilms grown in vitro, stained with LiveDead stain. Live bacteria stain green, dead ones red. The irregular silicone disc surface also takes up stain giving rise to a stained background in those images that show biofilms in situ. The paired images show the maximum intensity projection first at the top of the page, followed by a gallery of images in the lower part of the page representing different sections through the biofilm. Within each is contained a set of three images, the top right showing green staining only, the top left red only, and the bottom right showing the green and red overlaid on the same picture. Day 1 biofilms scraped off a disc and seen embedded in a matrix (A - max and B - gallery with 0.2μm steps). Fixed day 1 biofilm on disc showing bacteria that are dead (red) due to fixative (C - max, D - gallery with 0.2μm steps). Silicone discs with day 1 biofilm (E - max, F - gallery with 1μm step size), and day 5 biofilms (G - max, H - gallery with 1μm steps). Arrows indicate bacterial groups.
The presence of extracellular matrix was also examined using Alcian Blue (AB) stain. No polysaccharide was seen on 1 day old biofilms, but the 5 day old biofilms stained positively (Fig 8.3.1.7).

Fig 8.3.1.7: Alcian blue stain showing biofilm matrix on 5 day old *S aureus* (F2315) biofilms (x1000).

8.3.2 Demonstrating reduced susceptibility to antibiotics

Demonstration of reduced susceptibility to antibiotics is a key step in showing that a biofilm has developed. Therefore, one day old and five day old biofilms were exposed to antibiotics (at different concentrations in multiples of 10 above the minimum inhibitory concentration, MIC) for 1 day prior to sampling (Fig 8.3.2.1, 8.3.2.2, 8.3.2.3). Biofilms were less susceptible to antibiotics than planktonic bacteria, and even antibiotic levels at 10,000xMIC for 1 day could not eradicate biofilms.
Fig 8.3.2.1: Biofilm bacteria (one day old) are less susceptible to antibiotics than planktonic (S. aureus F2387). Clean control discs contained no bacteria.

Fig 8.3.2.2: Biofilm bacteria (five day old) are less susceptible to antibiotics than planktonic (S. aureus F2387). Clean control discs contained no bacteria.
Fig 8.3.2.3: Biofilm bacteria (five day old) are less susceptible to antibiotics than planktonic (S aureus F2315). Clean control discs contained no bacteria.

8.3.3 Processing of silicone discs for analysis

A number of methodological factors related to the model were investigated, using S aureus. It was important that any planktonic bacteria were removed prior to disc analysis. The biofilm had to be separated from the disc and bacteria freed to allow bacterial counting; trypsin and sonication were used to achieve this, but method development required that we chose the optimum way of achieving this without killing bacteria in the process.

*How many rinses are needed to remove planktonic bacteria?*

In order to assess effects of serial rinsing on bacteria we used a planktonic bacterial suspension in TSB placed in an Eppendorf tube for 5 minutes, and subjected to serial rinsing with 1mL sterile PBS (Fig 8.3.3.1). Whilst some bacteria continued to be obtained after rinses 2, 3, 4 and 5 (30, 140, 240, 155 CFU/mL respectively), the largest drop in CFU was observed after the second rinse (approximately 1000 fold drop in CFU). This suggests that two rinses
remove the vast majority of bacteria, and rising an additional 3 times does not result in "cleaner" samples.

For purposes of the biofilm model, three rinses were chosen when planktonic bacteria needed to be removed.

![Graph: Effect of serial rinsing](image)

Fig 8.3.3.1: Effect of serial rinsing on the number of bacteria obtained in each sequential rinse

**How long should sonication last for?**

Sonication uses ultrasound to disrupt the biofilm matrix and render bacteria planktonic so that they can be grown and counted on an agar plate. Biofilm discs, in 1 ml of PBS in an Eppendorf tube, were placed into a water bath at 37°C, and 50 Hz sonication applied (Precision Ultrasonic Cleaning, Ultrawave, Cardiff, UK). The resulting bacterial suspension in PBS was plated onto a sheep blood agar plate.
To determine appropriate sonication times to remove attached bacteria, the disc was sonicated for 5 minutes and the bacterial suspension plated, and the disc was then sonicated again to see if more bacteria could be removed from the disc; the different time periods thus relate to one disc. Sonication time of 5 minutes extracted the majority of bacteria (Fig 8.3.3.2), but 10 and 15 minutes also continued to extract a smaller number of bacteria (1075 and 220 CFU/mL, respectively). No bacteria were obtained after sonication for 30 or 45 minutes, suggesting that 15 min is an adequate sonication time to recover all attached bacteria (when sonication is used without trypsin).

Fig 8.3.3.2: Effect of sonication time on the number of bacteria obtained from biofilms growing on silicone discs. No bacteria were seen after 30 and 45 minutes.
Does adding trypsin before sonication increase bacterial yield?

Trypsin is a proteolytic enzyme that disrupts biofilm attachment to render bacteria planktonic. A range of trypsin (trypsin 0.25% with EDTA 0.02%, Sigma, Gillingham UK) incubation times prior to sonication was trialled and combined with different sonication times (Fig 8.3.3.3). In this experiment, rather than repeated sonication of a disc for increasing time periods (as in previous experiment Fig 8.3.3.2), each time period represents a different disc.

![Effect of trypsin and sonication](image)

Fig 8.3.3.3: Effect of trypsin and sonication times on bacterial yields from biofilms growing on silicone discs.

As in the previous experiment, in the absence of trypsin, 15 min sonication is most effective. However, combining trypsin with sonication shows that the highest yield is obtained with a 15 minute incubation in trypsin and sonication for 5 minutes (1.1 x 10^6 more CFU obtained than the nearest rival, and 3.5 x 10^5 more CFU than the greatest yield obtained with sonication alone in the absence of trypsin). Although overall the yield has changed by less than a
factor of 10, adding trypsin demonstrated a trend towards increased bacterial yield and therefore trypsinisation was incorporated into the protocol.

**Could trypsin be used without sonication?**

Bacterial counts were also performed using the finalised protocol of 15 min trypsin, single rinse, and 5 min sonication. Bacterial counts after sonication were greater than after trypsin alone (unpaired t test p=0.018) (Fig 8.3.3.4), confirming that sonication increases yield compared to trypsin alone.

![Graph: Can trypsin be used without sonication?](image)

**Fig 8.3.3.4:** Bacterial counts after trypsin only versus after addition of sonication.

**What is the effect of PBS rinse after trypsinisation?**

Two experiments were conducted to ensure that rinsing after trypsinisation (to remove the enzyme) does not remove a significant number of bacteria. Firstly, bacterial counts were performed on trypsin solution itself after 15 min incubation with the biofilm disc, then on the PBS after three rinses, and then
on the sonicated fluid. As expected the trypsin solution itself has a high number of CFU (Fig 8.3.3.5), which falls once trypsin is removed and replaced by PBS. Sonication increases CFU, but the overall difference in number is small (ANOVA \( p=0.169 \)). The finalised protocol also included only a single PBS rinse, and not 3 rinses as in this experiment.

![Trypsinisation, rinsing and sonication](image)

Fig 8.3.3.5: Bacterial counts in trypsin, after 3 rinses, and after sonication.

In a further experiment, rather than a rinse after trypsinisation, the trypsin solution itself was subject to sonication, and bacterial counts compared with those seen when one PBS rinse took place prior to sonication (Fig 8.3.3.6). This showed that performing a single PBS rinse after trypsinisation did not have a significant effect on the number of CFU (unpaired t test \( p=0.605 \)). This allowed incorporation of a single PBS rinse into the method to avoid leaving bacteria in trypsin for extended periods of time.
Effect of PBS rinse after trypsin

![Graph showing CFU/mL for trypsin only and with PBS rinse.]

Fig 8.3.3.6: Sonication in trypsin versus one PBS rinse before sonication.

*Does sonication affect bacterial viability?*

To ensure that sonication did not kill bacteria, a planktonic bacterial suspension was sonicated for varying time periods and plated (Fig 8.3.3.7). There was no evidence of sonication affecting bacterial viability, although experiments above indicate that longer sonication (or trypsinisation) times did not necessarily increase bacterial numbers obtained from the silicone disc. The rise in counts after sonication compared to the initial suspension probably represent a degree of bacterial clumping in the initial suspension and their dispersion with sonication.
Effect of sonication on bacteria

![Graph showing effect of sonication on bacteria](image)

Fig 8.3.3.7: Effect of sonication on bacterial viability.

**8.3.4 Antibiotics**

Antibiotics were added to achieve the final concentrations shown in Table 8.3.4.1. The lowest concentration was chosen as it represented the MIC of the *S aureus* used in the experiments tested on Etest strip (rifampicin typically 0.003 - 0.006 mg/L and clindamycin 0.064 - 0.125 mg/L), with remaining calculated concentrations increasing by a factor of 10.

<table>
<thead>
<tr>
<th>DOSE</th>
<th>RIFAMPICIN mg/L</th>
<th>CLINDAMYCIN mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1MIC</td>
<td>0.006</td>
<td>0.1</td>
</tr>
<tr>
<td>10MIC</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>100MIC</td>
<td>0.6</td>
<td>10</td>
</tr>
<tr>
<td>1,000MIC</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>10,000MIC</td>
<td>60</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 8.3.4.1: Antibiotic concentrations in biofilm models

*Do antibiotics remain effective for duration of experiment?*

To ensure that antibiotics remain effective for the duration of the experiment, 21 day old antibiotic and TSB solution used in biofilm eradication experiments
was mixed with Iso-Sensitest agar to make an agar plate containing rifampicin and clindamycin, at concentrations equivalent to the MIC for the test *Staph aureus* organism. The antibiotic mix was still effective 21 days into the experiment as no bacteria grew on the plate.

*Is there any antibiotic in washings or discs after detachment protocol?*

To ensure that no antibiotic is present on the discs themselves or in the washings after bacterial detachment protocol, the sonication solution from clean discs with 10,000MIC antibiotic dose was mixed with bacterial inoculum on a blood agar plate overnight. There was no difference in bacterial count between plates incubated just with bacteria, and those incubated with the addition of sonicated fluid.

*Does immediate addition of antibiotics to freshly-prepared discs inhibit bacteria, i.e. can antibiotics prevent biofilm formation / establishment?*

The effect of antibiotics on bacteria attached to discs for 2-3 hours was investigated by adding antibiotics to the models immediately after the discs were placed into bijoux bottles. Antibiotics at MIC levels were able to inhibit bacterial growth on such freshly-prepared discs.

### 8.3.5 Culture results and the LiveDead stain

A comparison of culture results and LiveDead stain results was undertaken (Table 8.3.5.1) on those samples that had both culture and LiveDead stain done as part of the assessment of antibiotic solution effectiveness on 1 and 5 day old biofilms (data and images shown in the section on biofilm eradication
using antibiotic solutions). The table shows that 7% of the culture-negative samples were LiveDead stain positive, and 4.8% of the LiveDead stain negative samples were culture positive. However, the Kappa value was 0.791, suggesting that the agreement was substantial. Taking culture result as the gold standard, the sensitivity of LiveDead stain was 88.2%, specificity 93%, positive predictive value 83.3% and negative predictive value 95.2%. If one assumes that the LiveDead stain is the gold standard, the sensitivity of culture was 83.3%, its specificity 95.2%. positive predictive value 88.2% and negative predictive value 93%.

<table>
<thead>
<tr>
<th>Culture</th>
<th>neg Count</th>
<th>pos</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>% within culture</td>
<td>93.0%</td>
<td>7.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within LD</td>
<td>95.2%</td>
<td>16.7%</td>
<td>71.7%</td>
</tr>
<tr>
<td>% of Total</td>
<td>66.7%</td>
<td>5.0%</td>
<td>71.7%</td>
</tr>
<tr>
<td>pos Count</td>
<td>2</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>% within culture</td>
<td>11.8%</td>
<td>88.2%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within LD</td>
<td>4.8%</td>
<td>83.3%</td>
<td>28.3%</td>
</tr>
<tr>
<td>% of Total</td>
<td>3.3%</td>
<td>25.0%</td>
<td>28.3%</td>
</tr>
<tr>
<td>Total Count</td>
<td>42</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td>% within culture</td>
<td>70.0%</td>
<td>30.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within LD</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>70.0%</td>
<td>30.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Table 8.3.5.1: Correlation between culture results and the LiveDead (LD) stain.
8.4 DISCUSSION

8.4.1 Summary of findings

Biofilms are defined as structured communities of bacteria attached to a surface and encased in self-produced extracellular matrix, and exhibiting reduced susceptibility to antibiotics compared to the planktonic bacteria from which the biofilm originates from (Parsek and Singh 2003). Any *in vitro* biofilm model, as a representation of a biofilm infection, therefore should contain both the physical and functional characteristics, with demonstration of reduced susceptibility to antibiotics essential (Donlan and Costerton 2002).

The data presented in this chapter show that biofilms can be established on silicone discs, and their presence confirmed by demonstrating both their structure and reduced susceptibility to antibiotics that typifies biofilms functionally. Five day old biofilms were chosen for further study. The optimum processing strategy to remove attached biofilms to render bacteria planktonic and thus allow their counting was also determined, with a final protocol of three PBS rinses, 15 minutes in trypsin, changing trypsin for PBS and then sonication for 5 minutes chosen.

8.4.2 Biofilm structure and reduced susceptibility to antibiotics

Rough silicone was chosen as the surface for biofilm attachment. Different surfaces vary in their biofilm susceptibility, but silicone has been found in most studies to be very conducive to biofilm development (Schinabeck and Ghannoum 2006) (Darouiche 2001) (Pascual 2002), whilst a rough surface
also enhances bacterial attachment (Schinabeck and Ghannoum 2006) (Darouiche 2001). Although biofilms formed in high shear environments may be stronger (Donlan and Costerton 2002), we used only a rotatory shaker for one hour, as the middle ear itself is unlikely to be exposed to significant shear forces.

**Biofilm structure**

The biofilm structure was demonstrated using a combination of SEM, confocal microscopy and Alcian blue polysaccharide stain. Environmental Scanning Electron Microscopy (ESEM) was also used, although the degree of hydration made the identification of individual bacteria in such images more difficult. Both SEM and ESEM image a sample by scanning it with a high energy electron beam in a raster pattern, with electrons interacting with the atoms in the sample to produce signals that give information about the surface of the sample, its composition and also electrical conductivity. However, in SEM the entire process is conducted in a vacuum, thus requiring the sample to be dehydrated. In the case of biofilms, this dehydration affects the matrix that contains a large amount of water, and the final images largely do not show much matrix. This does give rise to good photographs, as individual bacteria are easily seen. In the case of ESEM, technological modifications allow examination of hydrated structures by placing the specimen in a hydrated chamber rather than a vacuum; by these means the biofilm matrix can be preserved, but this means that individual bacteria are not seen as well because they are surrounded by matrix.
SEM gives surface characteristics of the bacteria, but LCSM is better at showing the three-dimensional nature of biofilms. LCSM works on the principle of optical sectioning, where images are collected point by point at selected depths and reconstructed using dedicated software (in conventional microscopy images from different depths are superimposed but in LCSM images from different depths can be portrayed separately). Thus LCSM allows one to determine the three-dimensional nature of biofilms by collecting images from different depths of a biofilm. A superimposed composite image showing maximum staining is also generated by the software. Using LCSM in conjunction with the LiveDead stain also allows the confirmation that bacteria in biofilms are alive.

Alcian blue has previously been used to stain biofilms and can also be used to quantify extracellular matrix production (Bayston and Rodgers 1990) (Bober 2005); it is a copper dye that reacts with polysaccharides to form an insoluble precipitate (Ramus 1977). The advantage of Alcian blue staining is that, because polysaccharide is an essential component of biofilm matrix (Lasa 2006), the stain should at least in theory detect the matrix of all biofilms. Various other stains for biofilm matrix can also be used, including a number of commercially available stains that can detect a variety of different matrix components (Invitrogen 2011).

Reduced susceptibility to antibiotics

In terms of reduced susceptibility to antibiotics, a difference could be seen between planktonic and biofilm bacteria after 24 hours of antibiotic treatment,
with biofilm bacteria not inhibited by any antibiotic concentration tested, but planktonic ones inhibited by antibiotics at a concentration of 100 times higher than the MIC of the inoculum. The reason for planktonic bacteria requiring 100MIC (rather than actual MIC level) probably lies in the fact that antibiotic treatment was carried out only for 24 hours. Rifampicin can be bacteriostatic or bactericidal depending on dose and exposure time, and exhibit time-dependent and also concentration-dependent killing (Gumbo, et al. 2007) (Bakker-Woudenberg, et al. 2005). Similarly, clindamycin can be bacteriostatic or bactericidal depending on dose and exposure time, and exhibit time-dependent (Klepser, et al. 1996) and also concentration-dependent killing (Aldridge and Stratton 1991). This is thought to explain the fact that a higher antibiotic concentration than MIC is required to inhibit planktonic bacteria at 24 hours. At longer incubation periods, even in the planktonic samples biofilm "slime" could be seen to develop at the bottom of a bijoux tube in the planktonic samples; thus data seen at longer time intervals are unlikely to represent pure planktonic bacteria.

**Effect of biofilm age**

Although communities of attached bacteria could be seen with SEM both in 1 and 5 day old biofilms, polysaccharide could be shown only on 5 day old biofilms. The observation that attached bacteria on both 1 and 5 day old discs exhibited greatly reduced susceptibility to antibiotics compared to planktonic bacteria suggests that attached bacteria develop recalcitrance to antibiotics before they start producing a significant amount of polysaccharide matrix, in keeping with previous literature (Otto 2008) (Dagostino, Goodman and
Marshall 1994). Later experiments on biofilm eradication (data shown in next chapter) showed that no great difference was seen when 5 day old biofilms were compared with 14 and 21 day old ones, suggesting that the *S. aureus* biofilm seen at 5 days was a mature one and therefore suitable for further studies involving a modified release antibiotic implant.

Our findings of the early development of reduced antibiotic susceptibility are comparable to other studies that have found biofilms forming as soon as at 24 hours (Vorachit, et al. 1993) (Nickel, et al. 1985) (Oliveira, et al. 2007), and that biofilm age is not necessarily a major factor influencing susceptibility to antibiotics (Wong, et al. 2010). This poses a potential problem when trying to eradicate biofilms, for if bacteria develop reduced susceptibility very early upon contacting a surface, then the higher antibiotic doses will be needed right from an early biofilm age. In the context of infections on prosthetic implants, this then becomes a major issue emphasising the importance of prevention of infection in the first place (for example with the use of antibiotic-impregnated biomaterials (Bayston, Fisher and Weber 2009) (Bayston, Vera and Ashraf 2010), although our findings that almost immediate addition of antibiotics to "infected" silicone discs (within a few hours and presumably before biofilms form) can prevent bacterial growth is consistent with the concept of peri-operative antibiotics as a way of minimising prosthesis infection.

In the context of OME though, the fact that biofilm age may be relatively unimportant could be an advantage. By the time children are treated biofilms
will probably have been present for months (at least 3 months if the NICE guidelines recommended 3 month waiting period is followed, and probably much longer as disease is likely to have been present months before clinical advice is sought). If biofilm age was a major stumbling block in biofilm eradication, then trying to eradicate biofilms that are months old would be difficult. However, if biofilms can be eradicated with biofilm age having a small role only, then the strategy of trying to eradicate OME biofilms could be a successful one. Certainly our results show that there is no significant difference in recalcitrance between biofilms aged between 5 and 21 days (also see Chapter 9), suggesting that eradication of biofilms several weeks old may be possible (providing appropriately high antibiotic levels are used for a long time period). We have not studied biofilms that are “months” old, but if reduced susceptibility develops on the first day, and there is not much difference in antibiotic susceptibility of biofilms within the first few weeks (Wong, et al. 2010), it may be tempting to speculate that eradication of “months” old biofilms may also be possible. However, the influence of biofilm age on its susceptibility to eradication remains an important issue.

8.4.3 Processing of biofilm discs

Biofilm bacteria attached to surfaces may be difficult to culture using standard laboratory techniques, but if the biofilm matrix is disrupted and bacteria rendered planktonic, then they should grow under standard laboratory conditions. This study therefore investigated different sonication times and trypsinisation times as a means of detaching bacteria from the silicone disc. On the whole, the differences between groups are smaller than a factor of 10.
Prolonged sonication and trypsin exposure (30 minutes) did appear to reduce bacterial yield, although tests on planktonic bacteria did not show any evidence of bacterial killing by sonication; previous work also suggests that ultrasound has little effect on Gram positive bacteria, but it inhibits many Gram negative ones (Monsen, et al. 2009). As trypsin is a proteolytic enzyme, prolonged exposure may affect bacterial viability, therefore a decision was made to remove trypsin after 15 min and replace with PBS before sonication (alternatives would have been to use a trypsin inhibitor, or to change pH or temperature to reduce trypsin activity). On the basis that the combination of 15 min trypsin and 5 min sonication yielded the highest count, that combination was chosen for future experiments. A number of methodological factors in the model were also investigated, so that we were able to establish that rinsing the discs three times removes majority of non-attached bacteria, that antibiotics remain effective for the duration of the experiments, and that no antibiotic remains on the discs after rinsing.

Although other studies have used broth turbidity (Ceri, et al. 1999) or in situ staining of bacterial biofilms with stains such as crystal violet (Djordjevic, Wiedmann and McLandsborough 2002) as a means of identifying bacteria, we chose to count colony forming units (CFU) as we felt that this represented a more accurate analysis of biofilm growth, particularly when one is looking at small numbers of CFU in resuscitation experiments. Therefore, bacteria had to be detached from the surface and rendered planktonic to allow CFU counting. Trypsin, used to aid detachment, is a proteolytic enzyme that has previously been utilised to remove biofilms from surfaces (Rocha, et al. 2008) (Chaignon, 170
et al. 2007), and is widely used in tissue culture to detach cells from a surface. It has also been used in an attempt to produce a biofilm-resistant surface by attaching trypsin enzyme to a stainless steel surface (Caro, et al. 2010). Sonication has also been widely used in biofilm detachment (Monsen, et al. 2009), and may also have future clinical applications as a possible diagnostic tool (Bonkat, et al. 2010) or an in vivo biofilm removal aid. We used an ultrasonic bath, where the generated ultrasound waves travel through water to create compression waves and local cavitation in contact with surfaces, leading to disruption of the biofilm's attachment to the silicone disc. Although we chose trypsinisation and sonication in our method, the processing of biofilms for analysis does vary in different methods / models, for example the well known Calgary biofilm device typically recommends only sonication to remove attached bacteria (Innovotech MBEC 2011).

We used rinsing to remove the antibiotic, as residual antibiotic could interfere with bacterial growth on agar plates following processing. This would be a particular problem if planktonic bacteria were plated with a solution containing antibiotics: although the antibiotic level may be too low to inhibit biofilms, it may be high enough to affect the planktonic bacteria newly-released from the biofilm. Our experiments indicated that the solution did not inhibit bacterial growth. An alternative to the rinsing would have been to incorporate an antibiotic neutralising step, as solutions that inhibit specific antibiotics are available, as is a universal neutralising solution (Innovotech MBEC 2011).
Although the two antibiotics would be unlikely to lose significant activity in 3 weeks, the continued effectiveness of antibiotics for the duration of the experiment was also examined, with no evidence that antibiotic effectiveness was lost. However, the method did not formally assay the concentration of antibiotics separately. This could have been done using HPLC or a biological method such as formal broth dilution (assaying the two antibiotics independently), but for the purposes of these experiments knowing that there is no significant deterioration of activity was felt to be sufficiently accurate. Clindamycin is very stable, but rifampicin can deteriorate if exposed to excess sunlight (Bhutani, Mariappan and Singh 2004), so the biofilm models and antibiotic solutions were kept away from direct sunlight.

8.4.4 Other biofilm models

Other established biofilm models are also available, the best known being the Calgary MBEC device (Ceri, et al. 1999) that allows high throughput analysis of biofilm grown on pegs suspended in a micro-titre plate (Fig 8.4.4.1), but the dimensions of the micro-titre plate wells are too small to be used as a middle ear model. Another commercially available device is the CDC biofilm reactor that is composed of discs (made of a variety of possible materials) suspended in a 1 litre flow chamber (Fig 8.4.4.2) (Goeres, et al. 2005), but the dimensions of this would again not be suitable as a middle ear approximation. Other in vitro models include the modified Robbins device where biomaterial samples are suspended in a flow chamber, and various models of biofilms grown on discs of glass or metal. As our aim was to develop a model in which we could test the antibiotic pellet, we needed to have something with the dimensions
that would allow the pellet to be placed into the nutrient broth together with the biofilm. This meant that the commercial Calgary device and CDC biofilm reactor were not suitable, and we had to develop a new model.

Fig 8.4.4.1 The Calgary device - MBEC™ high throughput assay (picture from manufacturer’s website) (Ceri, et al. 1999) (Innovotech MBEC 2011)
In addition to allowing the testing on antibiotic pellets with dimensions based on those of the middle ear, the model developed above is also useful because determination of bacterial counts is possible with the ability to detect only a small number of surviving bacteria. This is important when studying biofilm eradication, although in life one may expect the body’s immune system to deal easily with only a handful of bacteria so that the presence of a few surviving bacteria in vitro may be of limited clinical importance (although it may lead to resistance).

Although useful for the study of biofilm eradication with antibiotic pellets sized for the middle ear, the model described above is very labour-intensive. Each silicone disc is processed individually by hand, and with experiments typically consisting of more than a 100 discs at a time the amount of time required to process the discs is substantial. Thus the model does not lend itself to mass
study of biofilms, where the Calgary device has distinct advantages. A possible modification of our system would be to use multi-well plates with larger wells, for example the 24 well plates would accommodate just under 2 mL of fluid, although the addition of the disc and pellet would reduce the volume of nutrient broth. If multi-well plates were used, there would also be concern about contamination from well to well during rinsing and sonication, or even during incubation as the wells are not individually sealed like the bijoux bottles in our model (the amount of handling required in our model is also a risk for contamination, although after initial trials contamination did not appear to be a major issue). The Calgary device gets around this issue by having pegs in each well, but as at present it is available only with the small wells of a 96 well plate (typically less than a third of a millilitre) it could not accommodate antibiotic pellets. As an alternative to the silicone discs, one could have developed a Calgary "well and peg" type device but sized it for a larger well. It may also have been possible to "scale" up or down and use a bigger pellet in a bigger model or a smaller pellet in the Calgary device, but due to concerns about the effect that scaling might have this was not felt appropriate.

In terms of the in vitro model and processing of the silicone discs, an important consideration is applicability to other situations. Apart from the limitations of this model compared to others detailed above, the establishment of biofilms, timing, and processing that were developed in our laboratory for S. aureus on a specific scale can be considered relevant only to this situation. Investigations in other laboratories, with different scales and different bacteria
would necessitate validation and optimisation of the *in vitro* model in the same way.

An interesting aspect of *in vitro* biofilm study is the use of conditioning films composed of a variety of components (fibronectin, fibrinogen, fibrin, albumin, collagen, elastin, von Willebrand factor) (Schinabeck and Ghannoum 2006) (Pascual 2002). Some biofilm models use this as a means of coating an artificial surface to make it more conducive to biofilm attachment (Frade and Arthington-Skaggs 2010), but the presence of a conditioning film is not thought to be crucial for biofilm formation by other studies (Bayston, Ashraf, et al. 2007); it may be that its importance (or not) depends on the bacterial species, the surface, stress forces and nutritional provision. It is also interesting that the conditioning film may have clinical relevance, with a study of ureteric stents finding that the presence of certain proteins is highly associated with subsequent stent encrustation and biofilm colonisation (Canales, et al. 2009), and a study of dental caries finding that saliva as a conditioning film promotes biofilm formation (Shimotoyodome, et al. 2007). In the model described in this thesis the biofilms formed without the need for a conditioning film, therefore its use was not examined.

**8.4.5 Culture results and the LiveDead stain**

When assessing the presence of live bacteria, the samples can be analysed by microbial culture, but the LiveDead stain may also have a role, for example if biofilm bacteria will not grow on an agar plate they can at least be identified as live by the LiveDead stain. As culture and LiveDead stain theoretically test
the same thing, i.e. the presence of live bacteria, they could be expected to give very similar if not identical results. In fact 7% of the culture-negative samples were LiveDead positive, and 4.8% of the LiveDead-negative samples were culture-positive. The Kappa value was 0.791, suggesting the agreement was substantial (Kappa range of 0.61-0.80 is considered as substantial agreement, and values of 0.81 or more as almost perfect agreement) (Landis and Koch 1977). Further, there was no evidence that culture systematically under-estimated the number of bacteria detected by the LiveDead stain. The LiveDead stain does have a small rate of falsely labelling bacteria as live, as described in the discussion of OME aetiology section.

Early experiments analysed biofilms both with culture and the LiveDead stain, but as the data above indicates both can be considered to measure the same thing. The advantage of culture was of course that it gave a quantitative result (although the LiveDead stain could also have been adapted to be quantitative e.g. by counting the number of bacteria per microscope field). Due to concerns about a small risk of falsely labelling bacteria as live using LiveDead stain it was felt better to rely only on culture results rather than using LiveDead or indeed both culture and LiveDead staining when determining biofilm eradication.
8.5 BIOFILM MODEL: CONCLUSION

Although many biofilm models have been described, and commercially available kits exist, the specific plans to test biofilm eradication using an antibiotic pellet sized for the middle ear required the development of a new biofilm model. The model consists of biofilms grown on a silicone discs, with biofilm presence confirmed by showing the structure of biofilms as well as their reduced susceptibility to antibiotics. The best way of assessing biofilm eradication is to look for and count any remaining bacteria, therefore a strategy to detach biofilm bacteria and render them planktonic prior to counting had to be developed. A number of methodological parameters were therefore investigated, with the final method of biofilm detachment chosen to consist of trypsinisation for 15 minutes, change of trypsin to PBS and then sonication for 5 minutes, with plating of the resultant suspension.