UNIVERSITEIT GENT

FACULTY OF MEDICINE AND HEALTH SCIENCES

Academic Year 2013-2014

THE BIOFILM PARADIGM AS THE ELUCIDATION OF OTITIS MEDIA WITH EFFUSION

Ann-Sophie DE PAEPE

Edward LAMBERT

Promoters : Prof. Dr. Ingeborg Dhooge, MD, PhD,
Prof. Dr. Mario Vaneechoutte, PhD

Co-promoter: Dr. Helen Van Hoecke, MD, PhD

Dissertation presented in the 2nd Master year
in the programme of

Master of Medicine in Medicine

Foreword

This thesis was written in the context of our Master degree in Medicine at the University of Ghent. During the past two years, we had the privilege to meet and work together with a lot of different people, who each had their own influence on our work. Therefore, we would like to use this opportunity to thank everybody who supported and helped us.

First of all, we would like to thank our promoters, Prof. Dr. Ingeborg Dhooge, Prof. Dr. Mario Vaneechoutte and Dr. Helen Van Hoecke, for their guidance, encouragements and their critical view on this work. We enjoyed working together with them in a pleasant, friendly and open atmosphere.

Special thanks goes to Leen Van Simaey, Pieter Deschaght, Piet Cools and Jonas Van Belleghem, who helped us with the technical aspects of this work in the LBR (Laboratory of Bacteriological Research). We want to thank them too for the pleasant and friendly atmosphere.

For the use of the confocal light scanning microscope, we want to thank Prof. Dr. Bart Van De Kerckhove, Stijn Vanhee, Katia Reynvoet and Sophie Vermaut, for their trust, help and patience.

At last, we want to thank Prof. Dr. G. Van Maele, for his time, guidance and help with the statistical analysis of our results.

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List of abbreviations

ACK	Ammonium-Chloride-Potassium
ALB	Alkaline lysis buffer
AOM	Acute otitis media
BTS	Bacterial test standard
CHOC plates	Chocolate plates
CLSM	Confocal laser scanning microscopy
COME	Chronic otitis media with effusion
CO_2	Carbon dioxide
Cq	Cycle of quantification
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ENT	Ear nose throat
ET	Eustachian tube
EtOH	Ethanol
FISH	Fluorescence in situ hybridization
GUH	Ghent University Hospital
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
H. influenzae	*Haemophilus influenzae*
H. pylori	*Helicobacter pylori*
IVD	In vitro diagnostic
LBR	Laboratory for Bacteriological Research
MALDI-TOF/MS	Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy
McRAPD	Melting curve random amplified polymorphic DNA
MEE	Middle ear effusion
NaOH	Natrium hydroxide
NO	Nitrogen oxide
NTHi	Non-typeable *Haemophilus influenzae*
OME	Otitis media with effusion
M. catarrhalis	*Moraxella catarrhalis*
PCR	Polymerase chain reaction

PK	Proteinase K
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
S. aureus	*Staphylococcus aureus*
SDS	Sodium Salt
SNHL	Sensorineural hearing loss
S. pneumoniae	*Streptococcus pneumoniae*
TCA	Trichloroacetic acid
TOF	Time of flight
TSB	Tryptic soy broth
URTI	Upper respiratory tract infection

Abstract

Objective

Otitis media with effusion (OME) is a highly prevalent disease in children, but the pathogenesis is still not well understood. Culture negative but PCR positive results from middle ear effusion (MEE) samples have led to the hypothesis that biofilm structures are involved in the pathogenesis of OME. Though a lot of research is focused on biofilms, there are few *in vivo* studies that prove a relation between biofilm formation and OME. Trying to establish the role of biofilms in the pathogenesis of OME, this research focused on two main aspects. The first goal was to confirm the hypothesis that the adenoid acts as a reservoir for otopathogenic bacteria by demonstrating that the same bacterial species and genotypes were present in both the MEE and the adenoid. The second goal was to find evidence of the presence of biofilm structures in the middle ear effusions of children with COME.

Methods

MEE and adenoid samples were collected from 34 patients with COME who underwent ventilation tube insertion and adenoidectomy. Nasopharynx swabs were collected from 11 patients. A pilot study was conducted in 13 out of 34 patients in order to optimise the different techniques that would be used in the final study (culture techniques, identification techniques and fluorescence in situ hybridization (FISH)) and to identify the most frequent bacterial species present in the nasopharynx, the adenoid and the middle ear effusions (MEE). Some of the results of the pilot study were analysed together with data of the final study. The final study comprised 21 patients, in which culturing, genotyping and fluorescence in situ hybridisation (FISH) were performed. Samples of all 34 patients were cultured on CHOC-plates in aerobic and anaerobic conditions. Cultured bacteria were identified using matrix assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) and genotyped by melting curve random amplified polymorphic DNA (McRAPD).

Results

The MEE and adenoid samples were culture positive for *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* or *Staphylococcus aureus* in 22/34 patients (65%) and 23/34 patients (68%) respectively. *H. influenzae* and *S. pneumoniae* were the most frequently cultured bacteria in the MEE and adenoid samples respectively. In 19/22 patients (86%) the same bacterial species was found in MEE and adenoid/nasopharynx samples. In 63% of these cases, the same genotype was found. Of the samples of which both qPCR and

culture were performed, a culture negative but qPCR positive results was found in 23.6%. Live bacteria were detected in 10/10 studied samples with CLSM. In 5/10 (50%) samples, *H. influenzae* specific biofilm structures were observed.

Conclusion

The results of this study support the hypothesis that the adenoid indeed acts as a reservoir for otopathogenic bacteria and thereby facilitates infection of the middle ear. Secondly, the findings in this study indicate that biofilms, specifically consisting of *H. influenzae*, are indeed present in the middle ear effusions of children with OME. This leads to the conclusion that biofilms may play a crucial role in the pathogenesis of otitis media with effusion, which is important in the understanding of this disease and the development of potential future treatment options.

Abstract

Doelstelling

Otitis media met effusie (OME) is een zeer prevalente ziekte bij kinderen, maar de pathogenese van deze ziekte is nog steeds niet volledig opgehelderd. Cultuur negatieve maar PCR positieve resultaten van het middenoorvocht hebben aanleiding gegeven tot de hypothese dat biofilm structuren betrokken zouden zijn bij de pathogenese van OME. Hoewel veel researchprojecten zich richten op onderzoek naar biofilm, zijn er weinig studies gepubliceerd waarin men een verband heeft kunnen aantonen tussen de vorming van biofilms en het ontstaan/in stand houden van OME *in vivo*. Om dit verband aan te tonen, richtte dit onderzoek zich op twee belangrijke aspecten: enerzijds werd getracht aan te tonen dat het adenoid dienst doet als een reservoir voor otopathogene bacteriën, anderzijds werd gepoogd evidentie te vinden voor de aanwezigheid van biofilm structuren in het middenoorvocht van kinderen met COME.

Methodes

Middenoorvocht- en adenoidstalen werden gepreleveerd bij 34 patiënten met chronische COME die diabolo's geplaatst kregen en die een adenoidectomie ondergingen. Nasofarynx swabs werden afgenomen bij 11 patiënten. Een piloot studie werd uitgevoerd met stalen van 13 van de 34 patiënten om de technieken te optimaliseren die in de finale studie zouden gebruikt worden (kweektechnieken en fluorescentie in situ hybridisatie (FISH)) en om de meest voorkomende bacteriën in de nasofarynx-, adenoid- en middenoorvochtstalen te identificeren. Sommige resultaten van de pilootstudie werden geanalyseerd samen met de resultaten van de finale studie. De finale studie bestond uit 21 patiënten, waarbij kweek, genotypering en FISH werden uitgevoerd. Alle stalen van de 34 patiënten werden in cultuur gebracht op CHOC-platen onder aerobe en anaerobe omstandigheden. Gekweekte bacteriën werden geïdentificeerd met behulp van matrix assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) en werden gegenotypeerd met behulp van melting curve random amplified polymorphic DNA (McRAPD). Voor de kwantificatie van bacteriën, aanwezig in de klinische stalen, werd kwantitatieve polymerase chain reaction (qPCR) gebruikt. Het visualiseren van biofilm in situ gebeurde met behulp van FISH en confocale laser scanning microscopie (CLSM).

Resultaten

De middenoorvocht- en adenoidstalen waren cultuur positief voor *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* of *Staphylococcus aureus* in respectievelijk 22/34 patiënten (65%) en 23/34 patiënten (68%). *H. influenzae* en *S. pneumoniae* waren de meest frequent aanwezige bacteriën in respectievelijk de middenoorvochtstalen en de adenoidstalen. In 19/22 patiënten (86%) werd hetzelfde bacteriële species gevonden in zowel het middenoorvocht als in het adenoid/nasofarynx. In 63% van deze gevallen betrof dit ook hetzelfde genotype. Van de stalen die zowel gekweekt werden als voor qPCR gebruikt werden, werd een cultuur negatief maar een qPCR positief resultaat gevonden in 23.6% van de gevallen. Levende bacteriën werden gedetecteerd in 10/10 bestudeerde stalen met CLSM. In 5/10 (50%) stalen werden *H. influenzae* specifieke biofilm structuren geobserveerd.

Conclusie

The resultaten van deze studie ondersteunen de hypothese dat het adenoïd dienst doet als reservoir voor otopathogene bacteriën en daardoor infectie van het middenoor faciliteert. Daarnaast wijzen de resultaten van deze studie erop dat biofilmstructuren, specifiek bestaande uit *H. influenzae*, aanwezig zijn in de middenoorvochtstalen van kinderen met OME. Dit leidt tot de conclusie dat biofilms inderdaad een cruciale rol kunnen spelen in de pathogenese van OME, wat belangrijk is voor het volledig begrijpen van deze ziekte en de ontwikkeling van potentieel nieuwe behandelingen.

The biofilm paradigm as the elucidation of otitis media with effusion

Introduction

1. Definition

Otitis media with effusion (OME) is an inflammation of the middle ear in which a collection of serous or mucous fluid (middle ear effusion) is present in the middle ear cavity behind an intact tympanic membrane. Typically, there are no signs or symptoms of an acute infection (no pain, fever or malaise), but hearing loss is common. When the effusion is present for more than three months, it is defined as chronic otitis media with effusion (COME) (1-3).

2. Epidemiology

OME is the most common middle ear disease in young children, with a peak prevalence around the first and fifth year of life. It accounts for 25-30% of all cases of otitis media (2, 4). Although OME is known as a benign condition, characterized by a high percentage of spontaneous recovery, it is also the most common cause of primary care visits, referral for surgery and use of antibiotics during the first years of life. More than 80% of all children will experience one or more episodes of OME before reaching the age of four (1, 3, 4). The cumulative recurrence rate is 50% within 24 months (1). There is a great variation in prevalence of OME over time, with a high prevalence in winter and autumn and lower prevalence in summer. In data collected from 1980 till 1989, the prevalence of beta-lactamase producing *Haemophilus influenzae* and *Moraxella catarrhalis* in middle ear effusions from patients with OME increased significantly (3, 5). The great variation of prevalence rates among the worldwide OME reports, ranging from 1.3 to 61%, can be the result of different diagnostic methods being used, differences in population and race, antibiotic use and the vaccination against middle ear pathogens (6). The impact of vaccination is much debated. Analysis of the best available published articles on this matter shows no significant effect of pneumococcal vaccination in the prevention of OME.

Rarely, OME is also seen in adults after upper respiratory tract infection (URTI), after rapid changes in air pressure due to air travelling or scuba diving or in association with nasopharyngeal masses. The incidence of OME in adults, however, is much lower than in children (2, 4).

3. Pathophysiology

The etiology of OME is multifactorial and many different factors play a role in the pathophysiology of OME (7, 8). To cause inflammation of the middle ear, pathogens have to be able to adhere to the nasopharyngeal wall and reside there, to enter the middle ear cavity through the Eustachian tube (ET) and to overcome the defensive mechanism of the middle ear (7). This leads to the understanding that two main factors are implicated in the pathogenesis of OME: dysfunction of the Eustachian tube and immaturity of the immune system. In this respect, patients can develop OME as the result of an AOM (acute otitis media) or OME can develop *de novo*.

3.1 Bacteriology

H. influenzae, Streptococcus pneumoniae and *M. catarrhalis* are the most common pathogens implicated in OME (3, 9, 10). Bacteriology is discussed in detail in section 10.

3.2 Dysfunction of the Eustachian tube

Impaired function of the Eustachian tube can have multiple causes. Functional impairment can be caused by inflammation secondary to an URTI. These infections, caused by respiratory syncytial virus, parainfluenza, rhinovirus, influenza, enterovirus or adenovirus, also have deleterious effects on the mucociliary system of the ET, which facilitates the entry of bacteria in the middle ear through the Eustachian tube (4, 7). Once in the middle ear, the pathogens must be able to withstand and overcome the defensive mechanisms of the tubotympanum (anatomic and immunologic). Some subpopulations of children are more at risk to have a compromised function of the Eustachian tube and to develop OME: children with down syndrome, a cleft palate or other craniofacial anomalies have higher chance of anatomical impairment of the Eustachian tube and thus are at high risk for anatomic causes of OME (4). Mechanical obstruction of the Eustachian tube can be caused by adenoid hyperplasia (7).

The anatomy of the Eustachian tube of children is different from adults, which contributes to the increased incidence of otitis media in early childhood. In infants, the Eustachian tube is short, horizontally orientated and lacks stiffness (7).

3.3 Immature immune system

The normal tubotympanum is immunologically protected not only by the adaptive immune system but also by the mucociliary system and the secreted molecules of innate immunity.

The normal innate immune system acts by microbicidal peptides and proteins, such as lysozyme, lactoferrin and defensins that can be found on the epithelium lining of the upper airway. These antimicrobial components can selectively disrupt bacterial cell walls and membranes, sequester microbial nutrients or act as decoys for microbial attachment. Therefore, potential middle ear pathogens may reside in the nasopharynx without causing middle ear disease (7).

Waldeyer's ring, a group of mucosa-associated lymphoid tissue consisting of the tonsillae palatinae, tonsillae linguales, the adenoid, the tonsillae tubariae and the plicae tubopharyngicae, acts as a primary adaptive immune defense mechanism at the entry of the respiratory tract (7, 11). Lymphoid cells can recognize and destroy nasopharyngeal pathogens. They are also responsible for the production of effector and memory lymphocytes that migrate to neighbouring mucosal sites to act as a reinforcement of the local immune response. Nasopharyngeal secretions contain secretory antibodies (sIgA and IgM) that inhibit bacterial and viral adherence and reduce nasopharyngeal bacterial colonization.

In young children, this adaptive immune response is not well developed. Children may also lack secretory IgA or specific IgG2 antibodies against the capsular polysaccharides of *S. pneumoniae*, which makes them very vulnerable for this pathogen (7).

4. Risk Factors

Knowledge of environmental (extrinsic) and host (intrinsic) risk factors for the development of OME is important in identifying a child at risk. In this way, primary and/or secondary preventive measures can be taken to prevent complications or sequelae (1).

4.1 Extrinsic factors

Crowded living conditions:

A high number of family members and a high number of siblings in the family increases the risk for OME. Also day-care attendance increases the risk of developing OME. Children in day-care have three times more risk of developing OME then children cared for at home.

Day-care environment can be a forcing ground for bacterial resistance due to heavy antibiotic prescribing and re-circulating infections (2, 6, 7).

Season

In colder months, the incidence of otitis media increases (1).

Breastfeeding

There is a lower incidence of OM in breastfed children because of the presence of specific serum IgG antibodies to Non-typeable *Haemophilus influenzae* (NTHi) and P6, an NTHi outer membrane protein, which may facilitate protection against otitis media (OM). To achieve an optimal protective effect, the child must be breastfed for at least the first 11 months of life (12).

Pacifier use

When children are using a pacifier, the risk of OM increases. Two possible mechanisms have been proposed for this causal relationship. One possibility is that sucking on a pacifier increases the reflux of secretions from the nasopharyngeal cavity to the middle ear. Another hypothesis is the possibility that the use of a pacifier may induce alterations in dental structure, which causes dysfunction of the Eustachian tube. (1, 12, 13).

Passive smoking and pollutants

Passive smoking in children is associated with a minimally increased prevalence of OM. Even prenatal smoking has an effect.

Increase of CO_2 and NO levels, as indicators of pollution, is associated with OM (12).

4.2 Intrinsic factors

Age

The peak prevalence for OME is in the first and fifth year of life (2, 11). Up to 80% of children have been affected by the age of 4. Prevalence declines beyond 6 years of age (2).

Atopy

Recent studies support the hypothesis of the relation between OME and atopy. Nasal allergic inflammation leads to swelling and obstruction of the Eustachian tube. This condition disturbs the physiological mucociliary transport. Several studies have shown a predominance of eosinophils, T-lymphocytes and Th2-mediators in the middle ear effusions, providing evidence that the inflammation of the middle ear of OME patients can be allergic in nature (6). Allergy is by far a greater risk factor than other identified factors, conferring a 2- to 4.5-fold increased incidence of OME compared with the incidence of OME in non-allergic people (14).

Impaired Immunologic status

Children with congenital or acquired immunological deficiencies (such as immunoglobuline deficiencies, chronic granulomatous disease, AIDS, immunosuppressive drugs) are at risk for persistent OME (1).

URTI and Eustachian tube dysfunction

Viral infection of the upper respiratory tract (URTI) is a predisposing condition that influences the ability of bacterial pathogens to induce inflammation and invasion of the middle ear mucosa due to deleterious effects on the protective mucociliary system of the ET (2, 15). The relative risk for OME increases by up to 2.7 times in presence of URTI. Children with recurrent or ongoing URTIs are significantly more likely to suffer from OME. The impaired function of the Eustachian tube due to URTIs may increase the susceptibility to accumulation of fluid in the middle ear. Prevalence of OME is 7 times higher in children with URTI then in those without URTI. Prevention from URTI might be the first step of preventing OME (6).

Reflux

Pharyngo-laryngeal reflux may cause inflammatory changes in the Eustachian tube, which may disturb mucociliary clearance in the ET and thus may facilitate the entry of bacteria in the middle ear cavity (1, 16).

Race

Black, Hispanic and other racial groups are more unlikely to have OM than white children, probably due to underdiagnosis because of lack of medical care (12).

Genetics

There's an important role of candidate genes and gene polymorphisms in the development of OM. This is supported by twin studies, identification of polymorphisms involved in genetic susceptibility and genome linkage studies (12).

5. Symptoms

OME is not associated with clinical signs of an acute infection (no pain or fever). The most important symptoms are hearing impairment and potential discomfort from the presence of the middle ear effusion. In most cases, the middle ear effusion results in a conductive hearing loss. Occasionally it leads to sensorineural or mixed hearing loss as well. Studies show that sensorineural hearing loss (SNHL) may be caused by diffusion of toxins through the round window membrane (17). Inflammatory agents can also cause permanent or temporary threshold shifts in the cochlear basal turn. In other studies, microscopic inflammatory changes were seen, especially in the perilymph of the basal turn (2, 17). However, in the majority of cases there is a conductive hearing loss of 10–40 dB. Five to ten percent of the children with OME suffer from more severe hearing loss of 40–50 dB (11). Associated with this hearing impairment, children may encounter an impaired language development and problems with social interactions in case of prolonged duration of OME, especially important in at risk children (e.g. children with developmental delays) (7, 8, 11, 15).

6. Complications

Common complications of OME are tympanic membrane perforation and atelectasis of the tympanic membrane. Tympanic membrane perforation results in conductive hearing loss where the degree of hearing loss is correlated to the size and location of the perforation. In rare cases, recurrent or protracted OME can lead to cholesteatoma formation. Vestibular problems can also be reported in OME (18).

7. Clinical diagnosis

Diagnosis is made by otoscopy and age-appropriate audiologic testing (7). Common oto(micro)scopic findings in OME are dullness, loss of the light reflex and amber-gold coloration of the tympanic membrane due to the middle ear effusion. The negative middle ear

pressure results in a more horizontal appearance of the malleus by drawing the long process of the malleus medially. Retraction of the posterior pars tensa or retraction of shrapnell's membrane may be visible (7).

Otoscopy alone is only poorly predictive of the degree of hearing loss associated with the presence of MEE (middle ear effusion). Therefore, audiometry is essential as a marker for the impact of OME and the likelihood of resolution, because the severity of the initial hearing loss is a predictive factor in spontaneous resolution of the disease (7, 19). Tympanometry objectively assesses the mobility of the tympanic membrane and is as such a valuable aid in the diagnosis of OME (1). The results of a tympanometry are classified in patterns related to various pathological conditions involving the middle ear and eardrum.

The clinical findings on otoscopy combined with a B or C2 tympanogram, indicate that a middle ear effusion is present on the day of the examination. The hearing thresholds and systematic questioning about possible developmental effects are practical clinical tools to assess the medium-term persistence of the disease and help to provide some pointers to the need for intervention (7).

8. Prognosis

OME is ultimately self-limiting in the majority of cases. Approximately 50% of all OME cases resolve within 3 months and 95% within 1 year (2, 7). By the age of 6, most children will not have further problems. However, a large cohort study showed a correlation between middle ear disease and delayed language development up to 10 years of age (2).

9. Treatment

Management decisions in children with OME depend on the duration of the effusion, the laterality, presence and severity of associated symptoms and comorbidity e.g. developmental delay. Therefore, these features should be documented at each assessment of the child with OME. In uncomplicated cases of OME, the initial management of OME during the first three months after diagnosis, consists of 'watchful waiting' and the monitoring of hearing, as in 50% of all cases the disease is self-limiting within these first 3 months (2, 7). Oral antibiotics, antihistamines plus oral decongestants or mucolytics have not proven to be of any benefit in OME and can cause adverse effects. Antihistamines (especially first generation) can cause behavioural changes, seizures and blood pressure variability. Oral corticosteroids are unlikely to improve symptoms in OME on the long term and are associated with important side effects (e.g. growth retardation). Intranasal corticosteroids are also unlikely to be of benefit in case of

bilateral OME (1, 2). Recent studies showed no evidence of benefit of routine use of antibiotics for children up to 18 years with OME and furthermore hold potential risk of adverse effects and induce bacterial resistance. Nasal auto inflation of the Eustachian tubes (Otovent®) may produce benefit if used regularly, but compliance is often low (1, 2, 7). If the inflammation is the result of an underlying disease or condition, e.g. rhinitis, medical treatment can be considered.

When the effusion persists bilaterally for more than three months, surgical treatment should be considered. The standard surgical treatment for (C)OME is ventilation tube insertion (tympanostomy tubes). The principal benefit of tympanostomy tube insertion is the restoration of hearing and clearance of the fluid and the potential feeling of pressure. Tube insertion improves hearing levels with 6-12 dB on average (1).

Sequelae due to tympanostomy tubes, such as tympanosclerosis, focal atrophy and the formation of a shallow retraction pocket of the tympanic membrane, are common but are generally transient. Transient otorrhea occurs in 16% of patients in the early post-operative period and later in 26%. Chronic or recurrent otorrhea however is infrequent (1). Persistent tympanic membrane perforation is the most important complication after expulsion of the tympanostomy tubes (20).

In case of recurrence of OME after expulsion of previous tympanostyomy tubes and/or in case of associated complaints of upper respiratory tract obstruction due to adenoid hyperplasia or recurrent symptoms of rhinosinusitis due to adenoiditis, adenoidectomy is recommended (21). But even when the adenoid is not hyperplastic or obstructive with respect to the Eustachian tube, adenoidectomy is useful in the management of OME. The reason for this is that the adenoid may act as a potential reservoir for chronic infection (21, 22). Children with OME undergoing adenoidectomy have less time with effusion, better hearing, longer time to first recurrence and require less surgical re-interventions compared to those patients receiving only tympanostomy tube insertion (1). Combination treatment of adenoidectomy and tympanostomy tube insertion may be more effective than adenoidectomy alone (2). A possible sequela after adenoidectomy is the post-adenoidectomy haemorrhage. A primary post-operative haemorrhage has an incidence of 0.6%, secondary (after discharge) post-operative haemorrhage is very rare (7).

Myringotomy is not an effective treatment for OME, because the incision closes within a few days. Tonsillectomy has also not been shown to be effective in the treatment of OME (1).

10. Bacteriology

OME was previously thought of as a sterile inflammatory process, because bacterial cultures were often negative. It was elucidated that the persistence of pro-inflammatory cytokines played a key role in the initiation and perpetuation of inflammation. However, research with polymerase chain reaction (PCR) techniques proves that these 'sterile' effusions contain DNA of pathogenic bacteria, which remains present up to four weeks after treatment with antibiotics. Bacterial mRNA and proteins are also found in the effusions, which proves that bacteria remain metabolically active. These findings lead to the hypothesis that bacteria live in a specialised formation, called 'biofilm' (8, 23). *H. influenzae, S. pneumoniae* and *M. catarrhalis* are considered to be the most common pathogens implicated in OME, and all are capable of forming biofilms (3, 9, 10, 15, 23).

H. influenzae, S. pneumoniae and *M. catarrhalis* may reside in the nasopharynx of children in the first three years of life without causing illness. They occur in higher quantities in children prone to develop middle ear infection ('otitis-prone' children) (10). Previous studies tried to show a relation between the microflora in the nasopharynx and the bacteria that were cultured from middle ear effusions in OME, but a straightforward correspondence could not be found (24). *H. influenzae, S. pneumoniae, M. catarrhalis* and *S. aureus* are the most common otopathogens found on the adenoid surface and in the middle ear effusion of patients with OME (8).

In addition to the idea that otopathogenic bacteria form biofilms in the middle ear, research groups were also able to find proof of intracellular infection in middle ear mucosal biopsies of children with OME. It is known that otopathogenic bacteria, including *H. influenzae, S. pneumoniae, M. catarrhalis and S. aureus* are able to invade cells (e.g. adenoidal cells) and survive within cells *in vitro* (25).

H. influenzae utilises several adhesive factors which lead to colonisation and invasion of human epithelial cells, including adenoidal epithelium. Adhesins, pili and lipo-oligosaccharides play an important role in this matter. *S. pneumoniae* has been shown to invade broncho-epithelial cells and has been demonstrated *in vivo* in the middle ear mucosa of children with OME. *M. catarrhalis* has been shown to be able to colonise and invade pharyngeal epithelial cells and has been found specifically in adenoids and tonsils. Polymicrobial interactions between *H. influenzae* and *S. pneumoniae* may facilitate the invasion of epithelial cells. *S. aureus* has been demonstrated to cause intracellular infection in

patients with chronic rhinosinusitis and has been detected in biofilms on adenoid tissue in patients with chronic otitis media (15).

Despite these findings, it is still uncertain what the role of intracellular infection in the middle ear of children with OME is *in vivo.* The presence of bacteria intracellularly and in biofilms can play a role in the development of treatments that target both intracellular infection and bacterial biofilm, since both might contribute to the persistence and recurrence of infection. β-lactam antibiotics, often (erroneously) used for the treatment of OM(E), have a poor penetration in cells and so they have a limited effectiveness against intracellular bacteria (25). Other reasons for antibiotic resistance may be a result of the physical barrier formed by the extracellular biofilm matrix, but this resistance may also stem from the fact that oxygen and nutrient limitation within biofilms induces metabolic quiescence, which in turn reduces antibiotic effectiveness, since most antibiotics are most active against dividing bacteria (23, 26).

11. Biofilms

Biofilms are robust communities of surface-associated microbes that are held together by polymorphic extracellular matrix material (27). They were first observed and described in 1684 by Anthony van Leeuwenhoek but named as such only during the last decades. Van Leeuwenhoek was able to observe the vast accumulation of microorganisms in dental plaque (27). Biofilms are the preferred mode of existence of many microbial species, because of the fact that they are able to survive in hostile environments and to colonise new niches by various dispersal mechanisms (27, 28). Approximately 99.9% of bacteria in nature are thought to be attached to a surface in the form of a biofilm and at least 65% of all human infections, mostly chronic infections, involve biofilms (26, 29). Each year, biofilms cost Europe billions of euros in medical infections, equipment damage, energy losses and product contamination.

Biofilm can form on almost any surface in the environment, be it natural (plants, animals, human) or synthetic. They can grow on virtually any kind of substratum (e.g., rocks, books, statues, paintings on tissue, stone or wood) (30). They have been studied because of their resistance to many antimicrobials and to decontamination techniques. Medically they are relevant, because the human body is inhabited by microbes that can potentially contaminate medical devices and cause disease (27, 28). Bacteria in biofilm formation are recognised as the cause of a variety of human infections, including endocarditis, dental caries, lung deterioration in cystic fibrosis, chronic urinary tract infection, bacterial vaginosis, prostatitis,

and infections of prosthetic devices (29). On the other hand, biofilms play essential roles in the purification processes in wastewater treatment and therefore, they are also a promising and potentially sustainable solution to global energy and waste issues (27, 28).

11.1 Formation and differentiation

Dental plaque is the first biofilm form that has been studied with respect to its microbial composition and sensitivity to antimicrobial agents. It is now one of the best-studied biofilm models, displaying all of the typical characteristic features (27) .

Biofilms are described as a thin layer of bacteria encased in a self-produced hydrated matrix of polysaccharides and proteins, which adheres to implanted medical devices or surface tissues. It occurs due to a crosstalk phenomenon known as ‘quorum sensing’, a system of stimulus and response between bacteria correlated to population density (21, 26). The formation starts by reversible attachment of motile bacteria to the surface. The adhesion becomes irreversible with loss of motility and with elaboration of a glycocalyx by the bacteria. Growth continues by the division of sessile bacteria and recruitment of other bacteria from the environment, which constitutes a biofilm of glycocalyx-enclosed micro-colonies (21) [Figure 1]. These micro-colonies are bisected by ramifying water channels that carry bulk fluid into the community by convective flow, so that the bacterial cells inside the biofilm have access to nutrients and oxygen (31). The exchange of nutrients facilitated by this biofilm architecture enables biofilm communities to develop considerable thickness and complexity while keeping individual cells in optimal nutrient situations in many locations within the biofilm (32). Biofilm formation and biofilm detachment are under control of chemical signals of the same type that regulates quorum sensing. These regulatory molecules guide the formation of slime-enclosed micro-colonies and water channels and enable phenotypic differentiation through differential gene expression between the genetically identical cells (31).

Biofilm bacteria show increased resistance to antibiotics and mechanical removal. Cells in biofilms express a radically different set of genes from those expressed in the corresponding planktonic cells. There is no single biofilm phenotype, but gene expression in sessile communities goes through a whole spectrum of changes as the community matures, and the planktonic phenotype begins to emerge as the biofilm begins to shed mobile cells (31).

The extracellular matrix of a biofilm structure provides a physical barrier that enhances pathogen resistance to host defences such as opsonisation, lysis by complement and phagocytosis, but it protects against antibiotics too. Antibiotic resistance may thus be a result of the physical barrier formed by the extracellular matrix, but this resistance may also stem from the fact that oxygen and nutrient limitation within biofilms induces metabolic quiescence, which in turn reduces antibiotic effectiveness (23, 26). When residing in a biofilm formation, bacteria are thus resistant to common antibiotics, but during 'planktonic shedding', freely swimming bacteria can be killed even when the same bacterial species is involved.

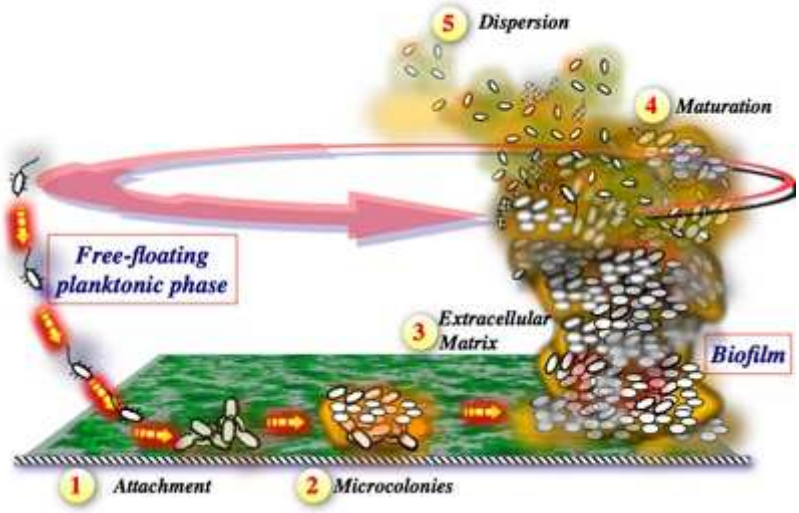

Figure 1: Principles of biofilm formation

Principles of biofilm formation picture [image on the internet]. 2006 . Available from http://www.pasteur.fr/recherche/RAR/RAR2006/Ggb-en.html.

11.2 Biofilm and OME

In middle ear mucosa of children with OME, biofilms of pathogenic bacteria have been found. The unique structure and characteristics of biofilm might explain the chronicity of the inflammation and its resistance to antibiotics (15, 21). In the OME effusion, the microorganisms are often difficult to culture and lead to a culture negative, but PCR- positive result (24). *H. influenzae, S. pneumoniae* en *M. catarrhalis* can be isolated in approximately 25% of children with OME, but PCR detects the presence of these pathogens in 80% of the children with OME (23).

Biofilms have been detected on adenoid tissue of groups of patients with OME (8). These biofilm structures may cause formation of biofilms in the middle ear, by a process called 'planktonic shedding' (8). The biofilm structure on the adenoid may disperse due to mechanical fragmentation or by release of single cells induced by cellular signalling. Biofilm fragments could thereby move to other areas (e.g. in the respiratory tract or through the Eustachian tube to the middle ear cavity), where they could cause an infection or form a new biofilm (8). The frequency of 'planktonic shedding' in biofilms is important, because it determines the coverage of mucosal surface and the frequency and activity of disease (8).

H. influenzae, S. pneumoniae, M. catarrhalis and *S. aureus* are all otopathogenic bacteria, able to form a biofilm structure. As mentioned above, they are also able to invade cells and reside intracellulary *in vitro*. The role of this mechanism in OME *in vivo* remains uncertain. The Nistico, *et al*. research group (15) found evidence that these bacteria reside in the adenoidal cells and form an intracellular biofilm in these cells. Therefore, both intra- and extracellular biofilms are formed in the adenoid, from where they can disperse into the middle ear cavity and form a new biofilm (8, 15). Polymicrobial interactions between *S. pneumoniae* and *H. influenzae* may facilitate epithelial cell invasion and have an effect on biofilm formation. Thus, co-infection results in more invasive bacterial strains. The elimination or persistence of these bacteria is influenced by both the host response and the competitive interactions between colonising microorganisms (3, 15).

In OME, biofilms may be attached to mucus as well as mucosa, thus providing the inflammatory stimulus leading to a middle ear effusion (3, 15). Available literature about biofilm in middle ear in children with OME is very poor, whereby some studies detected biofilm on the middle ear mucosa (23, 25), but biofilm in MEE has been investigated *in vivo* in only one study (3). In this study, 62 samples were taken from 42 patients, who were listed for ventilation tube insertion. Effusion samples were cultured on six different media, depending on type of bacteria. Confocal light microscopy (CLSM) was used to visualise biofilms. Twenty-eight of the 62 samples (45.2%) were culture-positive, cultured bacteria were *coagulase negative staphylococci, Veilonella species, Staphylococcus species, Streptococcus pneumoniae, Bacillus species*, *Moraxella catarrhalis,* and *Pseudomonas species.* Living bacteria were demonstrated in 51 samples (82.3%) by CLSM of which 49% showed biofilm (3). Biofilm formation has also been detected on adenoid and tonsil surfaces, particularly in children with recurrent infections (25). Fluorescence in situ hybridisation (FISH) and CLSM have proven to be good techniques to demonstrate the presence of multiple

bacterial species, including pathogenic bacteria associated with otitis media, intracellularly or in biofilms in the mucosal biopsies from children with OME. CLSM makes it possible to visualise hydrated specimens, while FISH allows determination of bacterial structures (25).

12. Conclusion

Though otitis media with effusion (OME) is a very common disease, representing 25-30% of all cases of OM and occurring in 80% of all children before the age of 4, the pathogenesis is still not well understood. Previously, OME was thought of as a sterile inflammation, but this hypothesis was invalidated by further studies with PCR techniques, which proved that bacterial DNA is present in the middle ear effusions. These bacteria may migrate from the adenoid, which may act as a potential reservoir for otopathogenic bacteria, to the middle ear cavity through the Eustachian tube.

The fact that middle ear effusions from children with OME are often culture-negative and the resilience of OME to antibiotic treatment has led to the hypothesis that bacteria associated with OME are organised in biofilms and/or reside intracellularly and thus cause intracellular infection. The actual role of these mechanisms *in vivo* remains undetermined.

13. General goals of the thesis

At present, there are not many studies that can prove a correlation between OME and biofilm formation in the MEE *in vivo*. Trying to find proof of the role of biofilms in the pathogenesis of OME, our research focused on two main aspects. The first goal was to confirm the hypothesis that the adenoid may act as a reservoir for otopathogenic bacteria, as suggested in some previous studies (15, 21, 22), by finding a relation between the bacterial species and genotypes present in both the MEE and the adenoid. The second goal was to find actual evidence of the presence of biofilm structures in the middle ear of children with OME.

This study was approved by the ethical committee of the Ghent University Hospital (Chairman: Prof. Dr. D. Matthys, Belgian registration number: B670201214394, date: 15/06/2012).

Materials and methods

1. Study population

Thirty-four children between 12 months and 6 years of age undergoing adenoidectomy and transtympanic ventilation tube placement for chronic OME (COME) were included in this study. COME is defined as persistent middle ear effusion without signs or symptoms of acute ear infection (fever, pain, discharge) for 3 months or more. The exclusion criteria were usage of local or systemic antibiotics within 30 days before the sample collection, known immune disorders, craniofacial malformation, previous adenoidectomy and participation in other clinical trials within the last 3 months before sample collection. Written informed consent was provided by the parents or the legal guardians of the study participants.

Clinical history of previous surgery in the ear nose throat (ENT) region (e.g. type and date of operation) and pneumococcal vaccination status were noted.

Environmental factors, such as type of day care/ school, number of children attending the day care/ number of classmates, number of siblings, duration of breastfeeding, pneumococcal conjugate vaccination status, passive smoking and usage of a pacifier were questioned.

2. Study setup

In order to perform this study protocol, the study had to be preceded by a pilot study. The goal of this pilot study, in which 13 patients were included, was to optimise the different techniques that would be used in the final study (culture techniques, identification techniques, FISH), to perform qPCR and to identify the most frequent bacterial species present in the nasopharynx, the adenoid and the middle ear effusion (MEE). Identification of the bacteria was performed after the collected samples were cultured as explained in *"methods"*.

The final study comprised 21 patients, in which culturing, genotyping and fluorescence in situ hybridisation (FISH) were performed. (See figure 2) Because of insufficient sample volume, qPCR was not performed in the final study.

For the interpretation of the results of this study, the results of both the pilot and the final study were combined.

3. Methods:

3.1. Surgical collection of MEE, nasopharynx swabs and adenoid tissue

Children included in this study were operated under general anaesthesia at the Ghent University Hospital (GUH). The outer ear canal was sterilised with 70% ethanol for 90 seconds. In a small pilot study, the effectiveness of the disinfection procedure was established.

The microflora of the outer ear canal of 10 patients (19 samples: 10 right ears, 9 left ears) was analysed after disinfection to control the effectiveness of the alcohol sterilisation. In 3 patients, both ears were sterile after disinfection. In 3 patients, one ear was sterile, while it was still possible to culture bacteria from the other ear. In 2 patients, both ears were culture positive after disinfection. In 2 patients, at least one ear was culture positive, but the bacteria could not be identified. The culture positive samples from outer ear canal swabs only consisted of a few small colonies of bacteria. Furthermore the cultured pathogens were mostly commensal skin flora and were not relevant for our research. Only 2 patients had an outer ear canal swab which was positive for possible otopathogenic bacteria (*Staphylococcus aureus* and *Turicella otitidis*). This finding can possibly be explained by the period of instillation. In our study this period lasted 90seconds while Daniel, *et al.* (3) instilled for 120 seconds and subsequently had culture negative results.

Middle ear effusions (when present) were aspirated through an incision of the drum using a Juhn Tym Tab. During surgery, the aspect of the middle ear effusion was scored as serous, mucous or purulent. After collection of the middle ear effusion, (a) tympanostomy tube(s) was/were inserted in the ear affected by COME. In 11 patients, nasopharyngeal cultures were collected with calcium alginate swabs during surgical intervention. In all patients, the adenoid tissue was removed using a curette.

The collected samples were sent to the Laboratory for Bacteriological Research (LBR), located at the GUH, within 1-2 hours.

3.2. Sample processing

The clinical samples were processed following a standard protocol. The bacteria in the collected MEE, nasopharynx swabs and the adenoid tissue were cultured on CHOC plates (i.e. agar plates to which boiled blood was added, in order to increase the nutrient availability for fastidious middle ear pathogens such as *Haemophilus influenzae*) according to the culture protocol (see infra: *3.2.1 Culturing*). The collected samples were used to make an easyMAG DNA extraction, which was then used for quantitative polymerase chain reaction (qPCR)

analysis. Genotyping was done with random amplified polymorphic DNA-analysis (RAPD) for those isolates that were cultured from at least two of the sampling sites.

The presence of biofilm in the MEE was investigated using fluorescent in situ hybridisation (FISH) in combination with confocal laser scanning microscopy (CLSM) (See Study flow chart, Figure 2).

Our research started with a pilot study in which bacteria were cultured on chocolate agar (CHOC)-plates and identified using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). When the same bacterial species was found in the MEE from both ears or in the MEE from at least one ear and in the adenoid tissue / nasopharynx, they were genotyped using McRAPD. Nasopharyngeal swabs were only collected in the pilot study, because the comparison of the culture results of adenoid and nasopharynx samples were mostly similar. Differences in culture results of these samples were considered to be the result of the sample collection technique (see Discussion - Pitfalls). Furthermore, the pilot study focused on the extraction of DNA directly from the clinical samples using the easyMAG equipment and protocol. This allowed us to collect all bacterial DNA from bacteria present in the adenoid tissue and MEE and perform qPCR on this DNA. These results were used to determine which culture and identification techniques were preferentially used in the final study.

In the final study, bacteria were cultured on CHOC-plates, identified using MALDI-TOF/MS and genotyped with RAPD when the same bacterial species was found in the MEE from both ears or in the MEE from at least one ear and in the adenoid tissue. EasyMAG DNA extraction was not performed to retain sufficient sample for FISH followed by CLSM which were performed to look for biofilm presence. (See study flow chart, Figure 2).

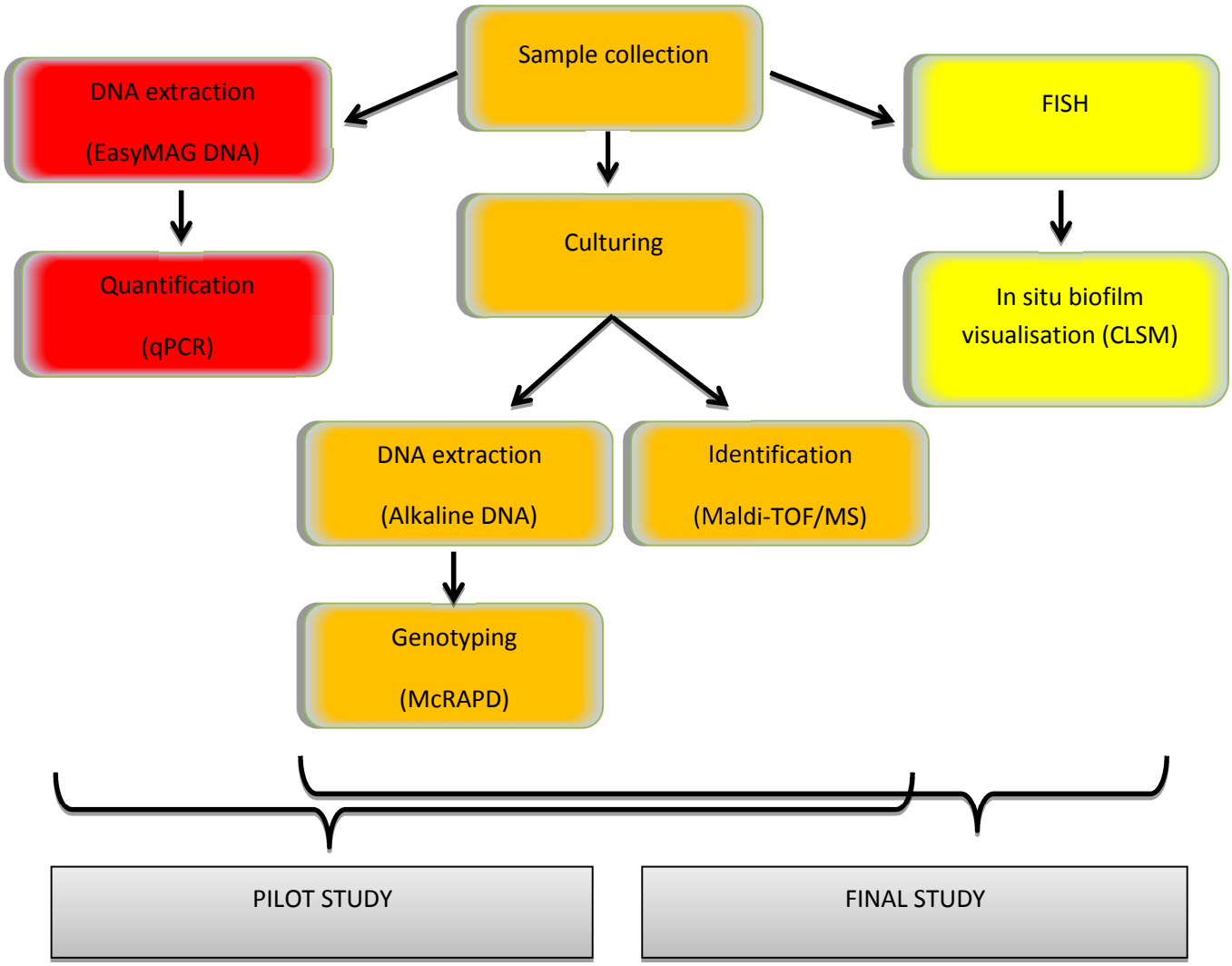

***Figure 2*:** Study flowchart

A. <u>Culturing</u>

1. <u>Culturing of the middle ear effusion (MEE)</u>

A 10 µl inoculation needle was used to inoculate CHOC-plates with MEE. These plates were then incubated aerobically with 5% CO_2 and anaerobically for 5 days.

2. <u>Culturing of nasopharynx swabs</u>

The nasopharynx swab was used to inoculate CHOC-plates. These plates were incubated aerobically with 5% CO_2 and anaerobically for 5 days.

3. <u>Culturing of the adenoid tissue</u>

The adenoid tissue was placed on an empty petri-dish and then cut in two equal pieces with a sterile scalpel. One piece was used for culturing and the other for DNA-extraction (see infra). The part used for culturing was divided into small pieces using a sterile scalpel. These small

pieces were collected, inoculated into 5 ml tryptic soy broth (TSB) and vortexed. This broth suspension was then incubated anaerobically at 37 °C for 10 minutes.

After 10 minutes, the broth suspension was vortexed again and 2 x 25 µl was inoculated onto two CHOC-plates. The broth suspension was then put back in the anaerobic incubator. The CHOC-plates were incubated aerobically with 5% CO_2 and anaerobically for 5 days. After 7 days, the broth suspension was vortexed again and 2 x 25 µl was inoculated onto two CHOC-plates, incubated as described above.

When multiple bacterial colonies grew on a CHOC-plate, representatives of those with different colony morphologies were re-isolated with a 1 µl inoculation needle onto new CHOC-plates following the same protocol as described above.

B. Identification

1. tDNA-PCR

1.1 General principles

Transfer RNA intergenic spacer length polymorphism analysis (tDNA-PCR) is a polymerase chain reaction (PCR) technique for identification of bacteria at the species or even subspecies level. The primers used in the PCR are based on conserved sequences located at the edges of the tRNA genes. Because the selected consensus primers are directed outwardly, the intergenic spacers are amplified rather than the genes themselves. With each PCR, several amplicons of different lengths are obtained, because several intergenic spacers, with different lengths, are present in each bacterial genome. Subsequent electrophoresis of the mixture of amplified tRNA spacer fragments yields electrophoresis patterns, consisting of multiple bands with different lengths. The patterns thus obtained (fingerprint) are rather conserved within a species and mostly different between species, although some variability exists within most species and some species cannot be differentiated from each other. As a result, tDNA-PCR can be used for identification of bacterial species.

Using one fluorescent primer, its incorporation during the PCR makes it possible to detect and visualise the fluorescent amplified fragments during high resolution (1 bp) fluorescent capillary electrophoresis on an ABI3130 machine. The amplicons are immediately digitised as tables composed of numerical fragment lengths (expressed in base pairs) and peak intensities. For identification, the resulting peak pattern can be compared with a large database of patterns of well-identified bacterial strains using a software package that is available online (http://www.basehopper.be).

1.2 Protocol

A PCR mix was made according to Table 1: 'PCR mix reagents' and 9.3 µl mix was pipetted into each well of a 96-well microtitre plate. This mix included a 1/5 dilution of fluorescent T3B-FAM primer (20 µM) in nonfluorescent primer T3B (20 µM), and of 20 µM of the non-fluorescent T5A primer. (Final reverse and forward primer concentrations: 0.2 µM).

In another pre-PCR room, 0.7 µl alkaline DNA extract from a cultured strain was added to the corresponding well. The plate was then covered with a PCR cap strip and put in the Veriti 96-well thermal cycler in the post PCR room. The tDNA-PCR protocol was run (see Table 2).

The amplified DNA-fragments were visualised and their length was determined by means of capillary electrophoresis.

Table 1: *PCR mix reagents for tDNA-PCR.*

Reagent	µl per bacterial strain	Final concentration
Polymerase supermix HiFi	9.1	1 x
Primer T3B + T3BFAM	0.1	0.2 µM
Primer T5A	0.1	0.2 µM
Alkaline DNA extract	0.7	NA
Total volume	10	NA

Legend: NA: not applicable

Table2: *Cycler program on Veriti thermal cycler.*

Phase	Time and temperature
Start	2' at 94 °C
PCR cycli (30x)	10" at 96 °C
	15" at 45 °C
	30" at 72 °C
Final extension	30" at 72 °C
Cooling	Permanent at 4 °C

2. Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight mass spectrometry (MALDI-TOF/ MS)

2.1 General principles

Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF/MS) is the most widely used method to date for the analysis of biomolecules. In recent years, it has been implemented in routine microbiology laboratories and has been utilised as a new approach for the identification of bacteria and yeasts (33).

MALDI-TOF /MS is based on the ionisation of co-crystallised sample material by short laser pulses, which creates ions of which the time of flight is measured in the vacuum flight tube of the equipment (33).

After the preparation of the target plate, as described below in 'protocol' (Figure 3B), the plate is inserted into the mass spectrometer (Figure 3C), where it is then transported to the measuring chamber. Within the machine, a high vacuum has to be continuously maintained. Once this vacuum has been created, the individual samples are exposed to short laser pulses. The energy of the laser vaporises the microorganism together with the matrix, leading to ionisation of the (predominantly ribosomal) proteins. These ionised peptides are then accelerated in an electromagnetic field, created by a potential of about 20 kV, before they enter the flight tube (Figure 4). The time of flight (TOF) of the analytes to reach the detector at the end of the flight tube is measured. The individual TOF is determined by the degree of ionisation as well as the mass of the proteins. Based on this TOF information, a characteristic spectrum is recorded and constitutes a specific sample fingerprint, which is unique for a given species (Figure 3D). For species level identification, the size range generally used is between 2 and 20 kDa. Computer software automatically compares the collected spectra with a reference databank containing a wide variety of medically relevant isolates (Figure 3E). The measured spectra are subject to method-inherent noise and therefore, will never be exactly identical for an individual isolate. The software which compares the spectra, generates a numerical value (score value) based on the similarities between the observed and stored data sets. This score value provides information about the validity of the identification. A score value above 2.0 is generally considered to be a valid species level identification. Values between 2.0 and 1.7 represent reliable genus level identifications.
After the analysis in the MALDI-TOF MS, the used target plate is removed from the equipment. The reusable target plate is cleansed with ethanol and trichloroacetic acid (TCA) solutions for further use (33).

The identifications obtained with the currently used MALDI-TOF MS techniques are nearly independent of culture conditions. Still, directly streaked colonies used for analysis should be as fresh as possible (not more than 48 h), because weaker and less distinguished peaks will appear in the spectra with increasing cultivation time. This effect is probably due to ribosomal protein degradation and leads to less efficient species identification (33).

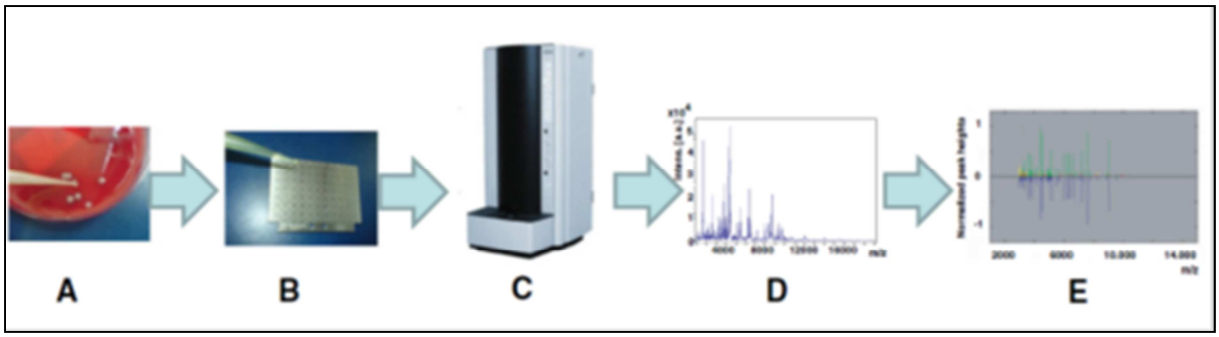

Figure 3: **A**: A single colony is taken from a culture plate. **B:** The target MALDI-TOF plate is prepared by spreading one colony over each cell of the target plate which is then covered with 1 µl of matrix. **C**: The target plate is inserted in the mass spectrometer. **D**: A specific sample fingerprint is generated based on time of flight (TOF) information. **E**: The fingerprint is compared with a reference database for identification (33).

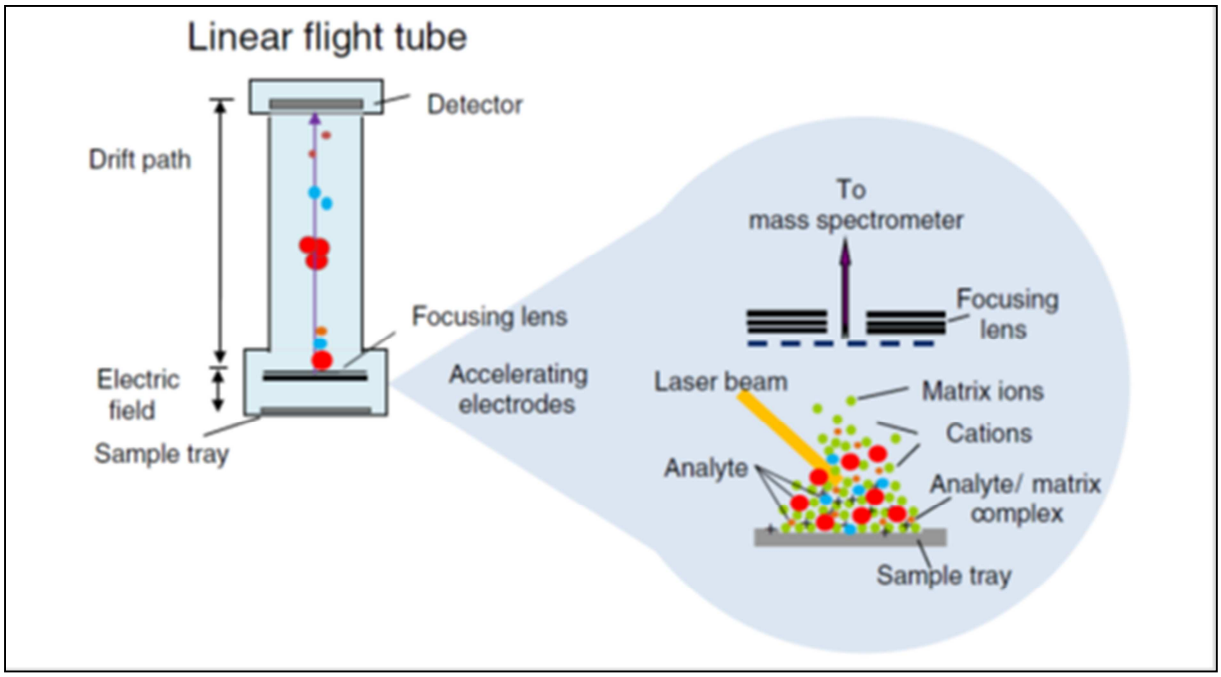

Figure 4: Principle of MALDI-TOF MS (33)

2.2 <u>Protocol</u>

MALDI-TOF/MS was used to identify bacteria from MEE and adenoid samples. These bacteria were cultured on CHOC-plates prior to analysis (as described above). We used the standard 'direct transfer' method for sample preparation with the in vitro diagnostic (IVD) procedure of the MALDI-biotyper.

From each of the plates with cultured bacteria, a colony was picked, using a 1 µL inoculation needle, and spotted evenly over the cells of the MALDI-TOF target plate. The preparations were covered with 1 mL of matrix solution (saturated solution of a-cyano-4-hydroxycinnamic

acid (α-CHCA) in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried for 2 min at room temperature. A bacterial test standard (BTS 255343, Brüker Daltonics, Germany) was used as positive control and an empty well covered with matrix served as negative control. Mass spectra were generated with a Microflex BiotyperTM spectrometer (Brüker Daltonics, Bremen, Germany), using the manufacturer's standard settings. For each sample, mass fingerprints were acquired, using Brüker Daltonics' flexControl version 3.0 software, analysed over a mass range of 2–20000 Da, and compared with the Brüker Daltonics' database.

C. Cryopreservation

A trypticase soy broth (TSB) + 15% glycerol solution was used as a maintenance medium for the cryopreservation of bacterial cultures. Glycerol acts as a cryoprotective agent providing intra- and extracellular protection against ice formation. Ice crystal formation is one of the main causes of freezing injuries.

To prepare this solution, 85 ml distilled water was pipetted in a sterile 250 ml bottle and 3 g TSB was added. Then, 15 ml glycerol was added and the bottle was autoclaved with open lid for 15 minutes at 121 °C.

Screw cap microtubes of 2 ml were filled with 1 ml of the TSB + 15% glycerol solution under a class II biological safety cabinet. A cotton swab was used to collect bacteria from CHOC-plates and was then rubbed against the bottom of the microtubes. These microtubes were labelled and stored at -80 °C.

D. DNA-extraction

1. General principles

As Figure 1 illustrates, two types of DNA-extraction were used. For quantification of the bacteria present in the clinical samples, pure DNA was needed, so easyMAG extraction was used. For identification of cultured strains with tDNA-PCR and genotyping of cultured strains with McRAPD, an alkaline DNA extraction was used.

2. Nucleic acid extraction by easyMAG

2.1 *General principles*

The NucliSens easyMAG nucleic acid extraction is a DNA extraction using magnetic silica particles and only works on effusion samples. The general principles of easyMAG extraction are summarised in figure 5. All cells, viral particles, bacteria and fungi are lysed by adding a chaotropic agent (guanidiniumthiocyanate), releasing the nucleic acids from the cells. The lysis buffer inactivates all nucleases present in the sample. The DNA is purified from the

contaminating proteins, sugars and lipids by adding magnetic silica to the lysed sample. Briefly, under high salt condition nucleic acid will bind to the silica particles. These silica particles act as the solid phase from which nonbound non nucleic acid components are removed by several washing steps performed in the NucliSens easyMAG instrument. Next, nucleic acids are eluted from the silica particles by using a low salt elution buffer. The resulting eluate contains purified and concentrated total nucleic acids.

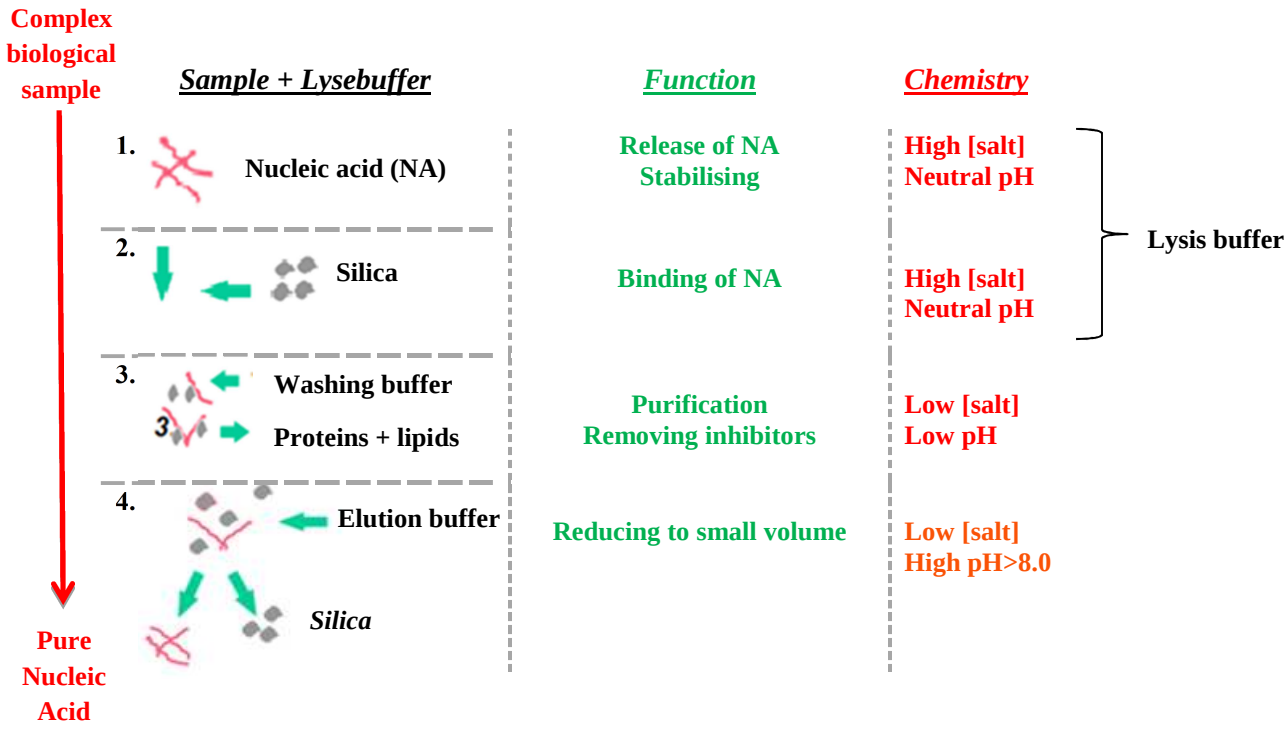

***Figure 5**: General principles of easyMAG DNA extraction*

2.2 Protocol

a. EasyMAG sample preparation for DNA-extraction from adenoid tissue

1. Cut the adenoid tissue in different pieces with a sterile scalpel (see: culture) and add one piece to a 2 ml tube.
2. Add 188 µl mutanolysine/proteinase K (PK) buffer.
3. Add 2 µl of 25 U mutanolysin/µl to the sample.
4. Liquefy the adenoid tissue with a tissuelyser.
5. Follow the protocol for easyMAG extraction for MEE starting from step 7 onwards.

b. EasyMAG sample preparation for DNA-extraction for middle ear effusion (MEE)

1. Add saline to the MEE until a final volume of 300 µl is reached.
2. Vortex.
3. Add 200 µl of this mix in a 2 ml tube.
4. Add 188 µl mutanolysine/proteïnase K (PK) buffer (*see B*).
5. Add 2 µl of 25 U mutanolysin/µl to the sample (50 units / sample)
6. Vortex.
7. Incubate for 15 minutes at 37°C.
8. Add 10 µl of a 25 mg/ml proteinase K solution (*see D*).
9. Vortex.
10. Incubate for 15 minutes at 55 °C, vortex every 5 minutes.
11. Add 1600 µl Nuclisens easyMAG buffer.
12. Incubate for 10 minutes at room temperature (RT).
13. Store at -80°C until extraction.

c. Mutanolysin/PK Buffer

Composition: 20 mM Tris-HCl, pH 8.0, 0.5% SDS

- 1 M Tris-HCl 1 l (stock in refrigerator 307**)**
    a. Dissolve 121.14 g tris(hydroxymethyl)aminomethane (MW = 121.14) in 800 ml H_2O
    b. Adjust pH to 8.0 with concentrated HCl.
    c. Adjust volume with water to 1 L.
    d. Make aliquots in 50 ml-falcons. Autoclave.
    e. Label with your name, content (1 M Tris HCl, pH 8.0) and date of preparation
    f. Store at 4 °C
- 20 mM Tris-HCl
    a. Transfer 1 ml of a 1 M Tris-HCl solution into a 50 ml Falcon tube.
    b. Adjust volume with water to 50 ml.
    c. Label with your name, content (20mM Tris HCl, pH 8.0) and date of preparation.
    d. Store at room temperature (RT).

- Mutanolysin/PK buffer
    a. Transfer 9.5 ml of a 20 mM Tris-HCl solution into a 14 ml Falcon tube.
    b. Add 0.5 ml of 10% SDS.
    c. Label with your name, content (20mM Tris HCl 0,5 % SDS, pH 8.0) and date of preparation.
    d. Store at RT.

d. Mutanolysin-solution

a. Dissolve 10000 U of mutanolysin (Sigma, Saint Louis, USA) in 400 µl of distilled mQ water.
b. Prepare aliquots of 20 µl each, labeled with Mut on the cap of the tube.
c. Store at -20°C in the box labeled with 'mutanolysin'.

e. Proteinase K solution

d. Prepare a 25 mg of proteinase K/ml stock solution by adding 4 ml of 20 mM Tris-HCl, pH 8.0 to 100 mg of proteinase K
e. Aliquot in 100 µl volumes
f. Label with PK
g. Store at -20 °C until use.

3. DNA extraction by alkaline lysis

The heater is pre-warmed to 95 °C. The alkaline lysis buffer (ALB*) has to be pre-heated in warm water bath or in a microwave until the precipitation has been resolved. When ALB is taken from an aliquot at room temperature and precipitation is not visible, pre-heating is not necessary. Tubes of 0.65 ml tubes are labelled according to the sample names. 20 µl ALB aliquots are pipetted into the 0.65 ml tubes. Next one small colony or part of a large colony is taken with a 1 µl inoculation needle and suspended in ALB in the corresponding tube. The tubes are heated during 15 minutes at 95 °C. After heating, the tubes are centrifuged a few seconds at maximum speed (16060 x *g*, 13000 rpm in Biofuge pico, Heraeus) to spin down the cellular debris. 180 µl HPLC-water is added to neutralise the pH. The tubes are centrifuged during 5 min at 16060 x *g*. Subsequently, the tubes are placed at – 20 °C during at least 30 min. The supernatant is used as the alkaline extract.

: Composition of ALB:

- 0.25% SDS
- 0.05 N NaOH
- 95 ml sterile UltraPure water (HPLC)

-Preparation of ALB:

1. Weigh 0.25 g SDS (Lauryl sulfaat (Sodium Salt) $C_{12}H_{25}O_4$, Sigma).
2. Add to sterile 100 ml bottle.
3. Add 95 ml sterile UltraPure water and 5 ml 1 N NaOH under flow.
4. Resolve precipitate in microwave or in heater at 95 °C until clear solution is obtained.
5. Filter sterilise with 0.45 µm filter.
6. Divide 10 ml into 1 ml aliquots in 1.7 ml cups.
7. Label as ALB with your initials and date (YY/MM/DD).
8. Store at room temperature.
9. Prior to dividing or to use: redissolve precipitate by heating as described above.

E. Quantification by quantitative PCR

Because the MEE samples were too small in volume, quantitative polymerase chain reaction (qPCR) and fluorescence in situ hybridisation (FISH) could not be performed both on the same sample. Therefore, only samples from patients included in the pilot study were used for qPCR.

1. General principles

qPCR is a DNA amplification technique based on the detection and quantification of a fluorescent signal which is directly proportional to the number of amplified DNA fragments (amplicons). The coupling of a thermocycler with a fluorimeter makes it possible to measure the fluorescent signal after each cycle of amplification (34). Because fluorescence increase is measured during the reaction, qPCR is also called realtime PCR (35). The ability of monitoring the amplification reaction during the exponential phase, makes it possible to determine the initial amount of target with great precision, by comparison to a standard dilution series containing a known number of initial genomes or target fragments. The cycle at which the fluorescence signal strength crosses the sensitivity threshold of the fluorimeter is called the “cycle of quantification” (Cq) value (34).

Fluorescent probes or non-specific fluorescent dyes (SybrGreen) can be used in qPCR (35).

- ***Non-specific fluorescent labels:***

Fluorescence can be incorporated in the amplicons by means of an intercalating fluorescent dye such as SYBR® Green I (SG) (34). SG has no fluorescence when it is free in a solution, but when it binds to the minor groove of the DNA double helix, it becomes fluorescent due to vibrations which convert electronic excitation energy into heat (35).

- ***Labeled sequence specific probes:***

It is also possible to use probes whereby the fluorescence of a fluorophore at the 3' end of the probe is inhibited by a quencher molecule at the 5' end of the probe (hydrolysis probes)(35). Probes bind to the previously amplified strands during the elongation step and subsequently, probe degradation occurs because the polymerase that is forming the complementary strand also has exonuclease activity. This frees the fluorophore from the quencher and as such fluorescence intensity doubles during each amplification cycle.

In this study, the following probes/dyes were used: species specific hydrolyis probes for quantification of *Haemophilus influenzae* and *Streptococcus pneumoniae* and SYBR Green for quantification of *Moraxella catarrhalis* and *Staphylococcus aureus*.

2. Protocol

- DNA extracts from clinical samples (MEE and adenoid) were prepared by easyMAG DNA extraction (see D: DNA extraction).
- In a pre-PCR room, a PCR mix was prepared:
    - For quantification with labeled sequence specific probes (used for *H. influenzae* and *S. pneumoniae*), the PCR mix contained the LC480 Probes Master, the species-specific hydrolysis probes and HPLC. 7.5 µl of this mix was pipetted in each well of the LightCycler480 plate.
    - For quantification with SG (used for *S. aureus* and *M. catarrhalis*), the PCR mix contained LC480 SYBR Green, two specific primers and HPLC. 8.0 µl of this mix was pipetted in each well of the LightCycler480 plate.
- In another pre-PCR room, 2.5 µl (in case labeled probes were used) or 2.0 µl (in case SG was used) of each DNA-extract was added to the mix.
- The plate was covered with sealing foil and centrifuged for 2 minutes at 1500 *g*.
- The plate was inserted in the LightCycler480 and the specific protocol for each species was run. In general, an amplification protocol started with denaturation at 95 °C during 5', followed by 40-45 cycles of 10" denaturation at 95 °C, 15" at the

annealing temperature of primers (and hydrolysis probe) and 1' elongation at 72 °C.

- Annealing temperatures were 55 °C for *H. influenzae* and *S. pneumoniae*, 50 °C for *M. catarrhalis* and 59 °C for *S. aureus*.

The primers used were hpdF729 and hpdR819, targeting the protein D gene of *H. influenzae*, in combination with hydrolysis probe hdpPrb727 [Wang, *et al.* 2012]. For quantification of *Streptococcus. pneumoniae*, we used primers lytA F373, lytA R424, targeting autolysin, in combination with hydrolysis probe lytA-probe [Wang, *et al.* 2012]. For *Moraxella. catarrhalis*, we used MCAT1 & MCAT2 in combination with SYBR Green [Post, *et al.* 1995]. For *S.. aureus*, we used femA-2F and femA-2R in combination with SYBR Green [Paule 2004].

F. Genotyping by melting curve random amplified polymorphic DNA (McRAPD)

1. General principles

3.1 McRAPD

When the same bacterial species were found in both the middle ear effusion and/or the adenoid, the strains were genotyped. To genotype the bacteria, genomic DNA was extracted by alkaline lysis and was used for melting curve random amplified polymorphic DNA (McRAPD). McRAPD is a PCR technique which does not require any specific knowledge of the DNA sequence of the target organism: random primers and/or primers directed against repetitive sequences will or will not amplify a segment of DNA, depending on positions that are complementary to the sequence of the primers. Thus, random segments of genomic DNA are amplified, which results in a strain specific fingerprint that can be used to distinguish different genotypes of the same bacterial species. In contrast to RAPD, which demands agarose gel electrophoresis to distinguish the different genotypes, melting curve analysis is used for McRAPD and instead of electrophoresis patterns, one obtains melting curve patterns. In our study, we used melting curve analysis for each McRAPD, but when the results were not obvious, agarose gel electrophoresis was added as a control.

3.2 Melting curve analysis

Melting curve analysis is a post-PCR analysis which determines the temperature at which the two strands of the amplified ds-DNA fragment(s) separate or melt. Because the DNA becomes single stranded when temperature rises, SYBR Green can no longer bind to this DNA fragment. The specific melting temperature of each amplified dsDNA strand depends

largely on the sequence, length, and guanine-cytosine (GC) content of the ds-DNA fragment.

The generation of melting curves is based on this principle: by slowly heating the PCR mixture after thermal cycling (i.e. the mixture of amplified dsDNA fragments) and measuring the decrease in fluorescence when SYBR Green is freed from the denatured DNA, a melting curve is generated. Different PCR-products with different lengths and base pair composition will have different melting temperatures. Thus, when fluorescence is plotted in function of time, these different PCR-products will have different peaks. Two strains of the same genotype will have the same peak pattern (36).

3.3 Agarose gel electrophoresis

As described above, McRAPD does not require agarose gel electrophoresis for interpreting the results. However, in our study, we conducted agarose gel electrophoresis when the results from melting curve analysis were not clear.

Using agarose gel electrophoresis, DNA fragments that vary in size can be separated. Agarose gel is a large-pored gel matrix that consists of agarose, a polysaccharide, which is added to an electrophoresis buffer. DNA samples, stained with ethidium bromide for visualisation under UV-light, are loaded into wells present in the agarose gel(37). Because of the presence of phosphate groups on DNA, these molecules are negatively charged at neutral pH. When an electrical field is put in place, these molecules thus migrate through the agarose gel towards the positive pole of the electric field. The speed at which DNA fragments migrate through the agarose gel depends largely on the DNA fragments' size. Small fragments will migrate faster than large fragments. Thus, DNA fragments with different sizes can be distinguished. By comparing the result with a marker with a known number of base pairs, the size of the DNA fragment can be estimated (37).

3.4 Protocol

To perform McRAPD, a mix of two primers (ERICII: AAG TAA GTG ACT GGG GTG AGC G and RAPD4: AAG AGC CCG T) and LC480 SYBR Green I master mix was made. 8 µl of this mix was pipetted into the cells of a PCR plate in a pre-PCR room. In another pre-PCR room, 2 µl of DNA extract of the strains to be genotyped, was added to the 8 µl of mix. The plate was then covered with a plastic film and centrifuged to bring down the mixes and to avoid air bubbles at the bottom of the wells at 476 x *g* (2200 rpm in Eppendorf 5430). The plate was inserted in the Lightcycler 480 (Roche Applied Science) and the following PCR program was run (Table 3) after the run, the PCR plate is stored at -20 °C.

Table 3: *Program of PCR run on LightCycler 480*	
Step	**Time & Temperature**
Denaturation	10’ 95 °C
Amplification (55x)	30” 95 °C
	20” 45 °C
	40” 72°C – single
Melting	5” 95 °C
	60” 60 °C
	97 °C – ramp 0.02 °C/sec – continuous
Cooling	30” 40 °C

G. Fluorescence in situ hybridisation (FISH)

1. General principles

FISH is a molecular cytogenetic technique in which the binding of fluorescently labelled DNA probes to their sequence complementary target can be visualised by fluorescence microscopy (i.e. also by CLSM) (38). The combination of these techniques can be used to visualise the morphology and spatial arrangement of targeted microbial cells, such as biofilm structures (39). FISH can be performed on many different targets, including RNA and DNA. For FISH on rRNA (which was performed in this study), microbial cells are permeabilised and incubated with fluorescently labeled oligonucleotides that specifically target rRNA (38, 39). DAPI staining, a universal EUB388-Alexa555 probe and a *H. influenzae* specific probe were used in this study.

2. Protocol

The ultimate purpose of performing FISH was to assess to what degree *H. influenzae* bacteria were present in biofilm structures in the MEE. The following protocol was used.

Day 1:

1. Place a heater at 80 °C and another heater at 50 °C.
2. Transfer 50 µl of MEE to a 0.6 ml Eppendorf tube.
3. Add 500 µl wash buffer and incubate for 5 min at room temperature.
4. Centrifuge 2 min at 11500 g.

5. Remove the Wash buffer and transfer the middle ear effusion to a new 0.6 ml Eppendorf tube.
6. Add 400 µl of Ammonium-Chloride-Potassium (ACK) lysis buffer.
7. Incubate at room temperature for 5 min, smoothly shake **(no vortex)** the 0.6 ml Eppendorf tube.
8. Centrifuge for 2 min at 11500 g.
9. Remove supernatant.
10. Repeat steps 6 to 9 until all the blood is removed from the sample
11. Add 500 µl Wash buffer and incubate for 5 min at room temperature.
12. Centrifuge 2 min at 11500 g.
13. Remove the Wash buffer.
14. Repeat steps 10 to 12.
15. Dehydrate the sample:
    a. Wash sample for 1 min in 300 µl 70 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    b. Wash sample for 1 min in 300 µl 85 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    c. Wash sample for 1 min in 300 µl 100% EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
16. Take one tube of 20 µl probe work solution (10 µM) and add 480 µl of hybridisation buffer (work solution 100nM) so the total volume becomes 500 µl, which is enough for 5 samples. Do this for the EUB388-Alexa555 and HAEINF probes.
17. Submerge the sample in 100 µl of the working solution of EUB388-Alexa555 and 100 µl of the working solution HAEINF probe in a 0.6 ml Eppendorf tube.
18. Incubate 5 min at 80 °C in heater, in the dark.
19. Incubate overnight in the dark (minimum 16 h) at 50 °C.

Day 2:

20. Place a heater at 73 °C.
21. Centrifuge sample for 2 min at 11500 g.
22. Remove supernatant.
23. Wash the sample with 300 µl Wash buffer with 3% Triton X-100 at room temperature for 4 min.
24. Centrifuge 2 min at 11500 g.
25. Remove supernatant.
26. Wash the sample with 300 µl Wash buffer with 3% Triton X-100 for 2 min at 73 °C.
27. Centrifuge 2 min at 11500 g.
28. Remove supernatant.
29. Wash the sample with 300 µl Wash buffer with 3% Triton X-100 for 2 min at room temperature.
30. Centrifuge 1 min at 11500 g.
31. Remove supernatant.
32. Dehydrate the sample in 0.6 ml Eppendorf tube:
    a. Wash sample for 1 min in 300 µl 70 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    b. Wash sample for 1 min in 300 µl 85 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    c. Wash sample for 1 min in 300 µl 100% EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
33. Add 200 µl of 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml dH_2O) to the sample in a 0.6 ml Eppendorf tube.
34. Leave the sample in the dark with DAPI stain on for 5 minutes at room temperature.
35. Centrifuge for 2 min at 11500 g.
36. Remove supernatant.
37. Wash the sample with 400 µl wash buffer for 2 min.
38. Centrifuge 2 min at 11500 g.
39. Remove supernatant.
40. Repeat steps 36 to 38.

41. Dehydrate the sample in 0.6 ml Eppendorf tube:
    a. Wash sample for 1 min in 300 µl 70 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    b. Wash sample for 1 min in 300 µl 85 % EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
    c. Wash sample for 1 min in 300 µl 100% EtOH.
        i. Centrifuge 1 min at 11500 g.
        ii. Remove supernatant.
42. Transfer sample to slide and divide the sample in two small pieces using a scalpel.
43. Transfer one of the small parts to a new slide.
44. Add a cover slide on each sample on the two individual slides.
45. Analyse the slides with the CLSM.

H. Confocal Laser Scanning Microscopy (CLSM)

CLSM is a microscopy technique used for 3D imaging of biological specimens. One or more lasers, which pass through an excitor, different dichromatic filters and a scanning unit, reach the objective lens of the microscope and hit the specimen. Scattered laser light and fluorescent light, formed by the excitation of the fluorescent markers, is detected by a photomultiplier tube, positioned just behind a pinhole, which restricts light that is out of focus or coming from above or below the plane of interest in the specimen. The output of the photomultiplier tube is processed by a computer and is then displayed as a digital image on a video monitor screen.

The CLSM produces optical sections of the specimen by scanning it point-by-point with the laser beam focused in the specimen, and using a spatial filter to remove unwanted fluorescence from above and below the focal plane of interest. Thus, only information from the focal plane of interest reaches the photodetector. As such, CLSM has the ability to produce multidimensional images. These images are created by the collection of images at different depths and/or time points(40).

Images produced by CLSM were processed and analysed using the ImageJ software package.

I. Statistics

Statistical processing of the results of this study was performed using the SPSS Statistics 20 software. To find a statistical correlation between two paired, dichotomous variables, a McNemar test was performed. In order to compare the results of this study with previous reported results, a Z-test for proportion was performed, which calculates a 95% confidence interval around a result $\left[p - 1.96\sqrt{\frac{p\,(1-p)}{N}}\,; p + 1.96\sqrt{\frac{p\,(1-p)}{N}}\right]$ (with p= the number of patients positive for a specific investigated characteristic and N= the population size).

When the results of other studies were not comprised within this confidence interval, this meant our results differed significantly ($p<0.05$).

Results

1. Patient characteristics, history and risk factors

In total, 34 patients were included in this study, of which 19 were boys and 15 were girls. Patient characteristics are summarised in table 4. The mean age of the included patients was 3.3 years old, with a maximum age of 6.6 years old and a minimum age of 1.1 year old.

Eleven out of 34 patients (33%) were not breastfed. In the group of children that were breastfed (66%), the mean duration of breastfeeding was 4.9 months, with a minimum of 1 month and a maximum of 12 months.

Table 4: *Patient characteristics*

		N
Sex	Male	19
	Female	15
Previous ENT surgery	None	27
	Bilateral tympanostomy tubes	6
	Facial nerve neurinoma	1
Pneumococcal conjugate vaccination	Not known	3
	Yes	29
	No	2
Type of daycare	Home	2
	Daycare <5 children	0
	Daycare >5 - <10 children	4
	Daycare >10 children	15
	School >=20 children	12
Number of siblings	0	9
	1	15
	2	9
Breastfeeding	Yes	22
	No	11
Passive smoking	Yes	1
	No	31
Soother use	Yes	11
	No	22

2. Culture

2.1 Pilot

In order to identify the most frequent bacterial species, a pilot study was performed. Samples of middle ear effusion (MEE), adenoid tissue and the nasopharynx from 13 different patients were used for culturing in this pilot study. In total, 23 MEE samples (11 left ears, 12 right ears), 13 adenoid samples and 11 nasopharynx samples were collected from these patients. After culturing these samples, 15 different bacterial species were found in the MEE samples, 45 different species in the adenoid and 21 different species in the nasopharynx. The results are shown in addendum I - Table I.

2.1.1 MEE

In the MEE, the most common bacterial species were *Haemophilus influenzae* (HI) (24%), *Staphylococcus epidermidis* (12.1%), *Staphylococcus aureus* (SA) (9.1%), *Staphylococcus caprae* (9.1%) and *Streptococcus pneumoniae* (SP) (9.1%). (See addendum I - Table I)

2.1.2 Adenoid

In the adenoid samples, the most common bacterial species were *S. pneumoniae* (10.5%), *H. influenzae* (8%), *S. aureus* (7%), *Actinomyces odontolyticus* (4.7%), *Granulicatella adiacens* (4.7%) and *Moraxella catarrhalis* (MC) (4.7%). (See addendum I - Table I)

2.1.3 Nasopharynx

In the nasopharynx samples, the most common bacterial species were *Cornynebacterium pseudodiphtheriticum* (15.2%), *H. influenzae* (13%), *S. epidermidis* (13%), *S. aureus* (10.9%) and *M. catarrhalis* (6.5%).(See addendum I - Table I)

Of all MEE, adenoid and nasopharynx samples together (164 samples), the most frequently found species were *H. influenzae* (12.8%), *S. aureus* (8.5%), *S. epidermidis* (7.9%), *S. pneumoniae* (8.5%) and *M. catarrhalis* (4.2%).

In different samples, multiple bacterial species were found. The mean number of species found in the adenoid, nasopharynx, right ear and left ear were 7, 4, 2.5 and 1.1 respectively. We did not receive a nasopharynx swab from 1 patient, a right MEE sample from 8 patients and a left MEE sample from 4 patients. There was no growth in 16 MEE samples. (See addendum I - Table II)

2.2 Final study

Because of the fact that *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and *S. aureus* were found in relatively high rates by culture techniques in the pilot study and are known to be otopathogens, we focused especially on these bacteria in the final study. Individual culture results from each patient included are discussed below in Table 6. Results below are combined with the pilot study.

2.2.1 MEE

In total, 57 MEE samples were collected from 34 patients, of which 41 samples (72%) were culture positive for bacteria. 29 MEE samples (51%) were culture positive for *H. influenzae, S. pneumoniae, M. catarrhalis* or *S. aureus*. 16 samples (18%) showed no growth.

When focusing on the number of patients, we can state that 29/34 (85%) patients had MEE with a culture positive result. In 22/34 patients (65%), the MEE of at least one ear was culture positive for *H. influenzae, S. pneumoniae, M. catarrhalis* or *S. aureus*. 5 out of 34 patients (15%) only had culture negative MEE.

The most frequently found bacterial species in the MEE samples was *H. influenzae*, which was found in 20 out of 57 MEE samples (35%).

2.2.2 Adenoid

All adenoid samples were culture positive for bacteria. 32 out of 34 adenoid samples (94%) were culture positive for *H. influenzae, S. pneumoniae, M. catarrhalis* or *S. aureus.*

In the adenoid, the most frequent bacterial species was *S. pneumoniae*, which was present in 23 out of 34 adenoid samples (68%). *H. influenzae* was present in 19 out of 34 samples (59%).

3. qPCR

Results from qPCR are shown in Table III in addenda. qPCR was only performed on the samples of the 13 patients from the pilot study. 7/12 (58.3%) MEE-samples, 11/12 (91.7%) adenoid samples and 5/5 (100%) nasopharynx samples were positive for *H. influenzae*. 3/11 (27.3%) MEE samples, 6/12 (50%) adenoid samples and 5/5 (100%) nasopharynx samples were positive for *S. pneumoniae*. Not one of the MEE samples (0/12) or adenoid samples (0/12) were positive for *M. catarrhalis*, but 2/5 nasopharynx samples were positive for *M.*

catarrhalis. 6/11 (55.5%) MEE-samples and 5/5 (100%) nasopharynx samples were positive for *S. aureus*, but all adenoid samples (0/12) were negative for this bacteria.

4. ***Relation between bacterial species in MEE and adenoid/nasopharynx samples***

Taking into consideration all 34 patients, 19 out of 22 patients (86.3%) with a culture positive MEE for one of the four bacterial species we focused on, the same bacterial species was found in the MEE of at least one ear and in the adenoid or nasopharynx. (See table 5) In 1/22 patients (4.5%), the same bacterial species was found only in the MEE of both the left and the right ear, but not in the adenoid/nasopharynx. In 2/22 patients (9%), no relationship between the bacterial species in the MEE and the adenoid/nasopharynx was found. In 2 patients, the MEE and adenoid/nasopharynx samples were culture positive for 2 different bacterial species. In 1 patient, these cultures were positive for 3 different bacterial species.

Table 5: *Results of culture for each individual patient, focusing on H. influenzae, S. pneumoniae, M. catarrhalis and S. aureus.*

Patient	**Nasopharynx**	**Adenoid**	**Right ear**	**Left ear**
ORL09	N	0	N	0
ORL10	SA	SA, SP	0	N
ORL11	MC	MC, SA	**HI**	**HI**, SP
ORL13	N	**SA**	**SA**, SP	0
ORL14	**HI**, SA	**HI**, SA	**HI**	0
ORL15	**HI**	**HI**	**HI**	**HI**
ORL16	HI, **SA**, SP	HI, SP	**SA**, Ss	0
ORL17	**SA**	HI, **SA**, SP	**SA**	N
ORL18	0	0	0	0
ORL19	HI	MC, SP	0	0
ORL20	**HI**, **SP**	**HI**, **SP**	**SP**	**HI**
ORL21	MC	**HI**, MC, SP	**HI**	0
ORL22	**HI**,MC	**HI**, MC, SP	**HI**	0
ORL23	/	**SP**	**HI**, MC, **SP**	**HI**
ORL24	/	**HI**, SP	**HI**	**HI**
ORL25	/	HI, MC	SP	NG
ORL26	/	**HI**, SP	**HI**	NG
ORL27	/	MC, SP	NG	NG
ORL28	/	HI, MC, SP	0	0
ORL29	/	HI, MC, **SP**	NG	**SP**
ORL30	/	**HI**, MC, SP	NG	**HI**
ORL31	/	**HI**, SP	**HI**	N
ORL32	/	SP	N	HI
ORL33	/	**HI**, MC, SP	**HI**, MC, SP	N
ORL34	/	**HI**	**HI**	**HI**
ORL35	/	HI, MC, SA, SP	N	NG

ORL36	/	SP	0	NG
ORL37	/	SP	NG	0
ORL38	/	HI, SS, MC	N	NG
ORL39	/	SP	N	NG
ORL40	/	**SP**	N	**SP**
ORL41	/	MC, SP	NG	0
ORL42	/	**HI, Ss**	**Ss**	**HI**, **SP**
ORL43	/	MC, **SA**	N	**SA**, Ss

Legend					
0	No relevant bacteria found	**N**	No sample	**/**	No test performed
NG	No Growth	**HI**	*H. influenzae*	**SP**	*S. pneumoniae*
MC	*M. catarrhalis*	**SA**	*S. aureus*	**Ss**	*Streptococcus species*
Bold	Relation between bacterial species		Relation adenoid/nasopharynx with left/right ear		

In 4/13 (30.8%) patients of the pilot study, the same bacterial species was found in a MEE sample and the adenoid or nasopharynx with qPCR. (See table 6) In these 4 patients, the relation between the bacterial species in the MEE and the adenoid/nasopharynx was also found by culturing.

Table 6: *Results of qPCR for each individual patient focusing on H. influenzae, S. pneumoniae, M. catarrhalis and S. aureus.*

Patient	**Nasopharynx**	**Adenoid**	**Right ear**	**Left ear**
ORL09	N	HI	N	SA
ORL10	N	0	/	N
ORL11	**HI**, MC, **SA**,SP	**HI**, SP	**HI, SA**	**HI**
ORL13	N	HI, SP	/	/
ORL14	**HI**, MC, **SA**,**SP**	**HI, SP**	**HI, SP**	**HI, SA, SP**
ORL15	HI, SA, SP	HI, SP	0	0
ORL16	**HI, SA, SP**	**HI, SP**	**HI, SA**, **SP**	**HI, SA**
ORL17	HI, **SA**,SP	HI	**SA**	N
ORL18	/	0	/	/
ORL19	/	HI	/	/
ORL20	/	HI	**/**	**/**
ORL21	/	HI, SP	**/**	/
ORL22	/	HI	**/**	/

Legend: See table 5

5. Genotyping

Genotyping was only done when the same bacterial species was found in both the adenoid/nasopharynx and the MEE of at least one ear or in the MEE of both ears (even when the adenoid was culture negative). Results from genotyping are shown in Table 7. In 12 /19 patients (63%) with the same bacterial species in both the MEE and the adenoid/nasopharynx, it involved the same bacterial genotype. In the 2 patients in which the same bacterial species was found in both the right and left MEE, but not in the adenoid/nasopharynx (ORL11 and ORL23, of which ORL23 also had a bacterial species (*S. pneumoniae*) that was present in the MEE and the adenoid/nasopharynx), it also involved the same genotype.

Table 7: *Results of genotyping isolates of the same species from different sites per patient*

Patient	**Species**	**Adenoid**	**Nasopharynx**	**Right ear**	**Left ear**
ORL09	/	/	N	N	/
ORL10	/	/	/	/	/
ORL11	HI	/	/	**a**	**a**
ORL12	/	/	/	/	/
ORL13	SA	**a b** b’	N	**a b**	/
ORL14	HI	**a** b	**a c**	**a c** d	/
ORL15	HI	a b	**c d**	**c**	**d** e
ORL16	SA	a b	/	c d	/
ORL17	SA	a b	0	c d	N
ORL18	/	/	/	/	/
ORL19	/	/	/	/	/
ORL20	HI	**a** b	c d	/	**a**
	SP	**a** b	**a**	**a**	/
ORL21	HI	0	/	/	a
ORL22	HI	a b c **d**	0	**d**	/
ORL23	HI	/	N	**a**	**a**b
	SP	a	N	b	/
ORL24	HI	**a** b c	N	0	**a** d
ORL25	/	/	N	/	/
ORL26	HI	**a b**	N	**a b**	/
ORL27	/	/	N	/	/
ORL28	/	/	N	/	/
ORL29	SP	a b c d	N	/	e f
ORL30	HI	**a**	N	/	**a**
ORL31	HI	**a** b	N	**a** c	N
ORL32	/	/	N	N	/
ORL33	HI	**a** b	N	**a** c	N
	SP	**a b**	N	**a b**	N
	MC	a	N	b	N
ORL34	HI	**a** b c d e **f**	N	**a** g	**f**
ORL35	/	/	N	N	/

ORL36	/	/	N	/	/
ORL37	/	/	N	/	/
ORL38	/	/	N	N	/
ORL39	/	/	N	N	/
ORL40	SP	a	N	N	b c
ORL41	/	/	N	/	/
ORL42	HI	**a**	N	/	**a**
	SP	**a**	N	**a**	**a**
ORL43	SA	a	N	N	b

Legend					
0	Negative	**SP**	*S. pneumoniae*	**HI**	*H. influenzae*
N	No sample	**MC**	*M. catarrhalis*	**Bold**	Relation adenoid-ear
/	No test performed	**SA**	*S. aureus*		

The relation between the presence of an identical bacterial species in the adenoid/nasopharynx and the middle ear on the one hand and the presence of the same bacterial genotype on the other hand was investigated. For this, only those patients who had positive culture results for both the MEE and adenoid/nasopharynx samples were selected (N=22). The relation between the presence of an identical bacterial species and an identical bacterial genotype on the places mentioned proved to be significant (McNemar Test, *p=0.03*).

6. ***Relation between culture and qPCR (pilot + final)***

The relation between culture and qPCR for adenoid samples is shown in Table 8.1. Samples from 12 patients were tested for the four most common otopathogens and compared. A similar result was found in 26/48 adenoid samples (54%) with culture methods and qPCR. In 8/48 (17%) of the adenoid samples culture was negative but qPCR positive. In the adenoid samples, 14/48 (29%) were culture-positive and qPCR negative. qPCR of the adenoid for S. aureus was negative for all samples, while culture results were positive for S. aureus in 7/12 (54%) of the samples.

The relation between culture and qPCR results for nasopharynx samples is shown in table 8.2. Samples of 5 patients were tested for the four most common otopathogens. In 11/20 (55%) of the nasopharynx samples, a similar result was found with qPCR and culturing methods. In 9/20 samples (45%), culture results were negative, but qPCR was positive.

The relation between culture and qPCR for MEE samples is shown in Table 8.3 (left ear) and 8.4 (right ear). Eleven samples from 7 different patients (5 left ear, 6 right ear) were tested for the four most common otopathogens and compared. The right MEE sample of ORL10 was tested only for *H. influenzae* and *M. catarrhalis.* A similar result was found in 30/42 cases (71%) with culture methods and qPCR. In 9/42 cases (21%), culture results were negative but qPCR was positive. In 3/42 cases (7%), culture results were positive but qPCR was negative.

For a total of 110 cases (including MEE samples, adenoid and nasopharynx samples tested for the 4 most important bacteria), both qPCR and culture were performed. In 26 of these cases (23.6%), there was a culture negative but qPCR positive result. This percentage proved to be not significant (McNemar Test: p=0.117). In 17 of these cases (15.5%), there was a culture positive but qPCR negative result.

When looking only to the results of the MEE samples, qPCR and culture were performed for a total of 42 cases. In 21.4% of these cases, there was a culture negative but qPCR positive result. This number proved to be not significant (McNemar Test: *p=0.146*).

Table 8.1: *Relation between culture and qPCR (Adenoid)*

	H. influenzae		***S. pneumoniae***		***M. catarrhalis***		***S. aureus***	
	Culture	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**
ORL9	**0**	**X**	**0**	**0**	**0**	**0**	**0**	**0**
ORL10	**0**	/	**X**	/	**0**	/	**X**	/
ORL11	**0**	**X**	**0**	**X**	**X**	**0**	**X**	**0**
ORL12	/	/	/	/	/	/	/	/
ORL13	**0**	**X**	**0**	**X**	**0**	**0**	**X**	**0**
ORL14	**X**	**X**	**0**	**X**	**0**	**X**	**X**	**0**
ORL15	**X**	**X**	**0**	**X**	**0**	**0**	**X**	**0**
ORL16	**X**	**X**	**X**	**X**	**0**	**0**	**X**	**0**
ORL17	**X**	**X**	**X**	**0**	**0**	**0**	**X**	**0**
ORL18	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
ORL19	**X**	**X**	**X**	**0**	**X**	**0**	**0**	**0**
ORL20	**X**	**X**	**X**	**0**	**0**	**0**	**0**	**0**
ORL21	**X**	**X**	**X**	**X**	**X**	**0**	**0**	**0**
ORL22	**X**	**X**	**X**	**0**	**X**	**0**	**0**	**0**

Legend: See table 8.4

Table 8.2: *Relation between culture-qPCR (Nasopharynx)*

	H. influenzae		*S. pneumoniae*		*M. catarrhalis*		*S. aureus*	
	Culture	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**
ORL9	N	N	N	N	N	N	N	N
ORL10	0	N	0	N	0	N	X	N
ORL11	0	X	0	X	X	X	0	X
ORL12	/	/	/	/	/	/	/	/
ORL13	N	N	N	N	N	N	N	N
ORL14	X	X	0	0	0	0	X	X
ORL15	X	X	0	X	0	0	0	X
ORL16	X	X	X	X	0	X	X	X
ORL17	0	X	0	X	0	X	X	X
ORL18	0	/	0	/	0	/	0	/
ORL19	X	/	0	/	0	/	0	/
ORL20	X	/	X	/	0	/	0	/
ORL21	0	/	0	/	X	/	0	/
ORL22	X	/	0	/	X	/	0	/

Legend: See table 8.4

Table 8.3: *Relation between culture-qPCR (Left ear)*

	H. influenzae		*S. pneumoniae*		*M. catarrhalis*		*S. aureus*	
	Culture	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**
ORL9	0	0	0	0	0	0	0	X
ORL10	N	N	N	N	N	N	N	N
ORL11	X	X	X	0	0	0	0	0
ORL12	/	N	/	N	/	N	/	N
ORL13	0	/	0	/	0	/	0	/
ORL14	0	X	0	X	0	0	0	0
ORL15	X	0	0	0	0	0	0	0
ORL16	0	X	0	0	0	0	0	X
ORL17	N	N	N	N	N	N	N	N
ORL18	0	/	0	/	0	/	0	/
ORL19	0	/	0	/	0	/	0	/
ORL20	X	/	0	/	0	/	0	/
ORL21	0	/	0	/	0	/	0	/
ORL22	0	/	0	/	0	/	0	/

Legend: See table 8.4

Table 8.4*: Relation between culture-qPCR (Right ear)*								
	H. influenzae		***S. pneumoniae***		***M. catarrhalis***		***S. aureus***	
	Culture	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**	**Culture**	**qPCR**
ORL9	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**
ORL10	**0**	**0**	**0**	**/**	**0**	**0**	**0**	**/**
ORL11	**X**	**X**	**0**	**0**	**0**	**0**	**0**	**X**
ORL12	**/**	**X**	**/**	**0**	**/**	**0**	**/**	**X**
ORL13	**0**	**/**	**X**	**/**	**0**	**/**	**X**	**/**
ORL14	**X**	**X**	**0**	**X**	**0**	**0**	**0**	**0**
ORL15	**X**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
ORL16	**0**	**X**	**0**	**X**	**0**	**0**	**X**	**X**
ORL17	**0**	**0**	**0**	**0**	**0**	**0**	**X**	**X**
ORL18	**0**	**/**	**0**	**/**	**0**	**/**	**0**	**/**
ORL19	**0**	**/**	**0**	**/**	**0**	**/**	**0**	**/**
ORL20	**0**	**/**	**X**	**/**	**0**	**/**	**0**	**/**
ORL21	**X**	**/**	**0**	**/**	**0**	**/**	**0**	**/**
ORL22	**X**	**/**	**0**	**/**	**0**	**/**	**0**	**/**

Legend					
	Culture positive-qPCR negative		Culture negative-qPCR positive		Correspondence culture- qPCR
0	Negative	**X**	Positive	**N**	No sample
/	No test performed				

7. *Fluorescence in situ hybridisation (FISH)*

FISH was performed on 14 MEE samples to detect biofilm formation, specifically by *H. influenzae*. Of these 14 MEE samples, 11 were culture positive for *H. influenzae.* Three other samples were used as a negative control. Of these 3 control samples, 2 were culture negative and 1 showed bacterial growth by culturing, but was not culture positive for *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* or *S. aureus.* In 14 of the 14 MEE samples, blood was visually present before the FISH protocol was run. In 4 of these samples (of which 1 negative control), blood was still visible after the protocol was run. FISH analysis was not possible for these samples.

The FISH results are summarised in table 9. In all 10 MEE samples, live bacteria were seen with CLSM. In 5 of the 10 MEE samples (50%) that were appropriate for FISH analysis, evidence of the presence of *H. influenzae* specific biofilm structures was found. (See figures 5-8).

In 2 out of 5 MEE samples in which no *H. influenzae* specific biofilm was detected, nonspecific bacterial clusters were found. In none of the negative controls, *H. influenzae* specific biofilms structures were detected. In 1 negative control (ORL37R), non-specific bacterial clusters were detected. Figures of samples in which *H. influenzae* specific biofilm structures could not be detected, are added in addenda (See addendum II - figures I & II).

Table 9: *FISH results*

Sample	Culture positivity for *H. influenzae*	Presence of blood in sample before protocol	Presence of blood in sample after protocol	Presence of live bacteria	Presence of *H. influenzae* specific biofilm structures
ORL23R	Yes	Yes	No	Yes	Yes
ORL24R	Yes	Yes	No	Yes	Yes
ORL24L	Yes	Yes	No	Yes	No
ORL26R	Yes	Yes	No	Yes	No
ORL30L	Yes	Yes	Yes		
ORL31R	Yes	Yes	No	Yes	Yes
ORL32L	Yes	Yes	No	Yes	Yes
ORL33R	Yes	Yes	Yes		
ORL34R	Yes	Yes	Yes		
ORL34L	Yes	Yes	No	Yes	Yes
ORL42L	Yes	Yes	No	Yes	No
ORL30R	No	Yes	Yes		
ORL37R	No	Yes	No	Yes	No
ORL37L	No	Yes	No	Yes	No

Legend	
	Sample not appropriate for FISH analysis due to presence of blood in the sample

General info figures 5-8: Three probes were used for the visualisation of biofilms in MEE samples. DAPI stained nucleoli blue. The EUB388-Alexa555 probe was a universal probe which stained bacteria green. The *H. influenzae* specific probe stained *H. influenzae* bacteria red. The combination of the EUB388-Alexa555 probe and the *H. influenzae* specific probe leads to a yellow colour, which specifically indicates the presence of *H. influenzae*.

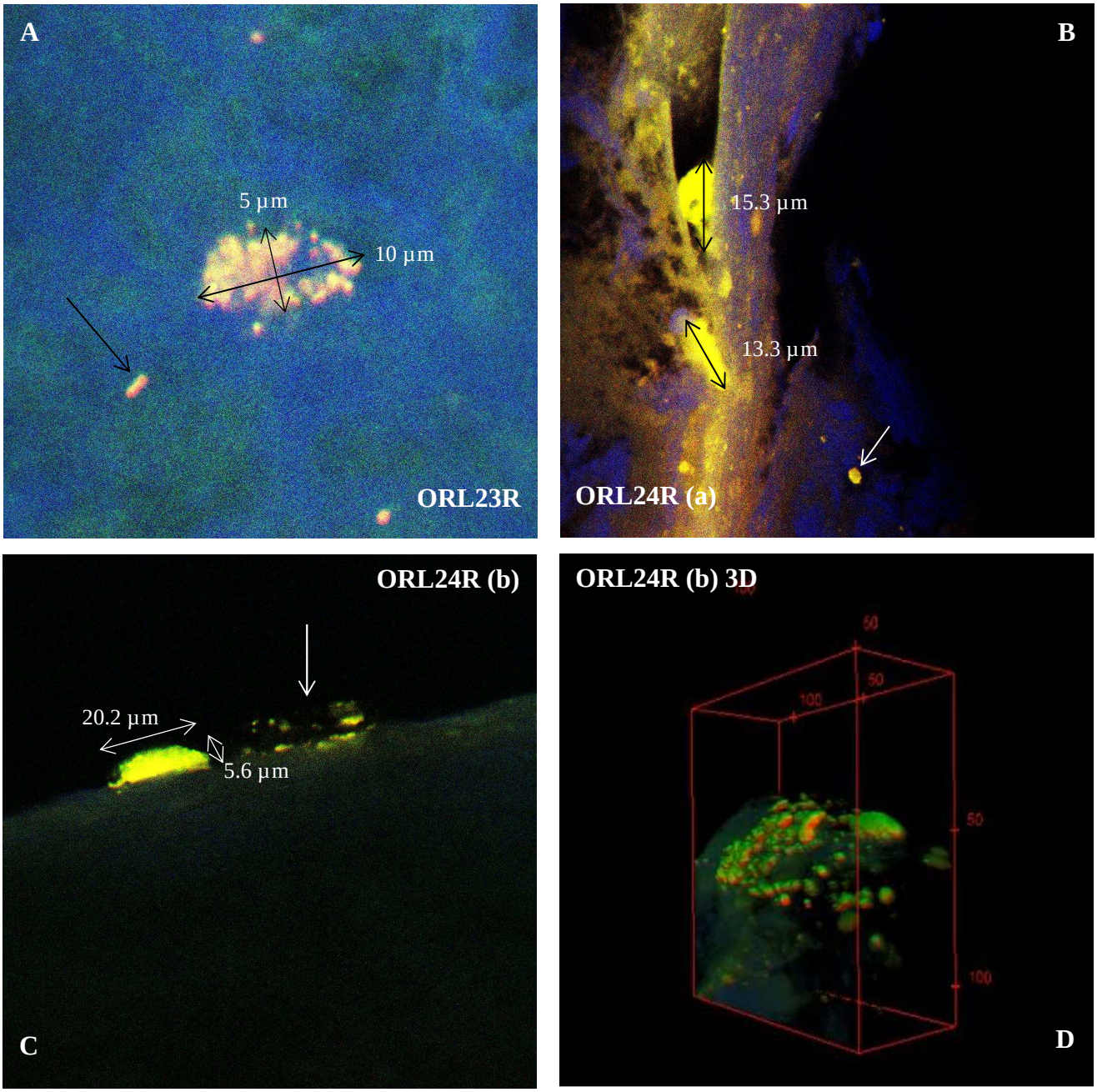

Figure 5: CLSM images of biofilm structures visualized by FISH. **(A)** A *H. influenzae* biofilm structure of 10 µm with planktonic - free floating *H. influenzae* bacteria around the biofilmstructure (arrow). **(B)** Two neighboring *H. influenzae* biofilm structures of 15.3 and 13.3 µm with planktonic – free floating bacteria from the biofilm structure (arrow). **(C)** A section of a larger biofilm structure visualised in 3D in **(D)** with planktonic – free floating bacteria around the biofilm structure (arrow).

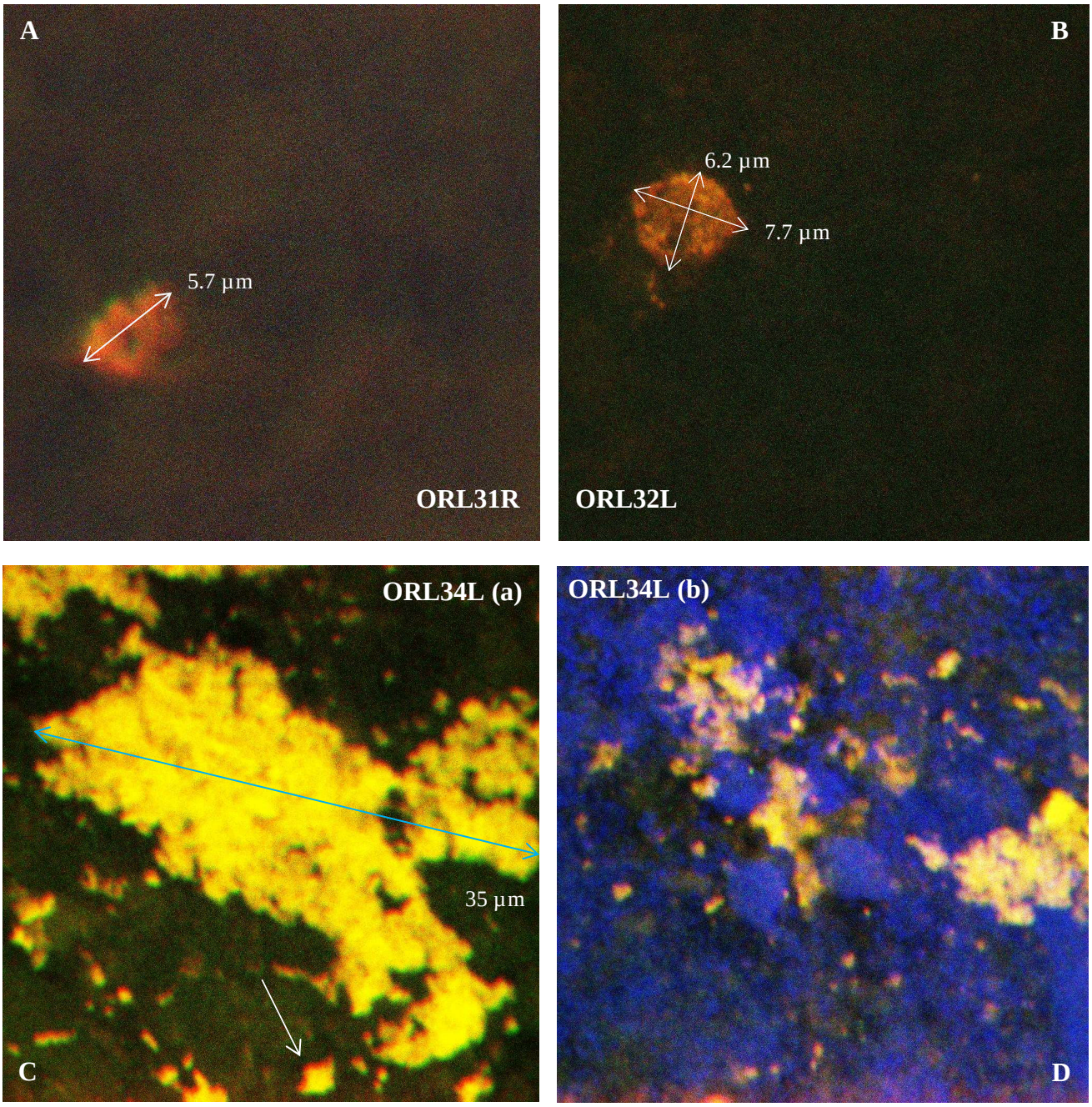

***Figure 6**:* CLSM images of biofilm structures visualized by FISH. **(A)** A biofilm structure of *H. influenzae* bacteria of 5.7 µm. **(B)** A biofilm structure of *H. influenzae* bacteria of 6.2 by 7.7 µm. **(C)** A large biofilm structure of *H. influenzae* bacteria of 35µm with planktonic – free floating bacteria around the biofilm structure (arrow). **(D)** A *H. influenzae* biofilm structure around DAPI stained nucleoli.

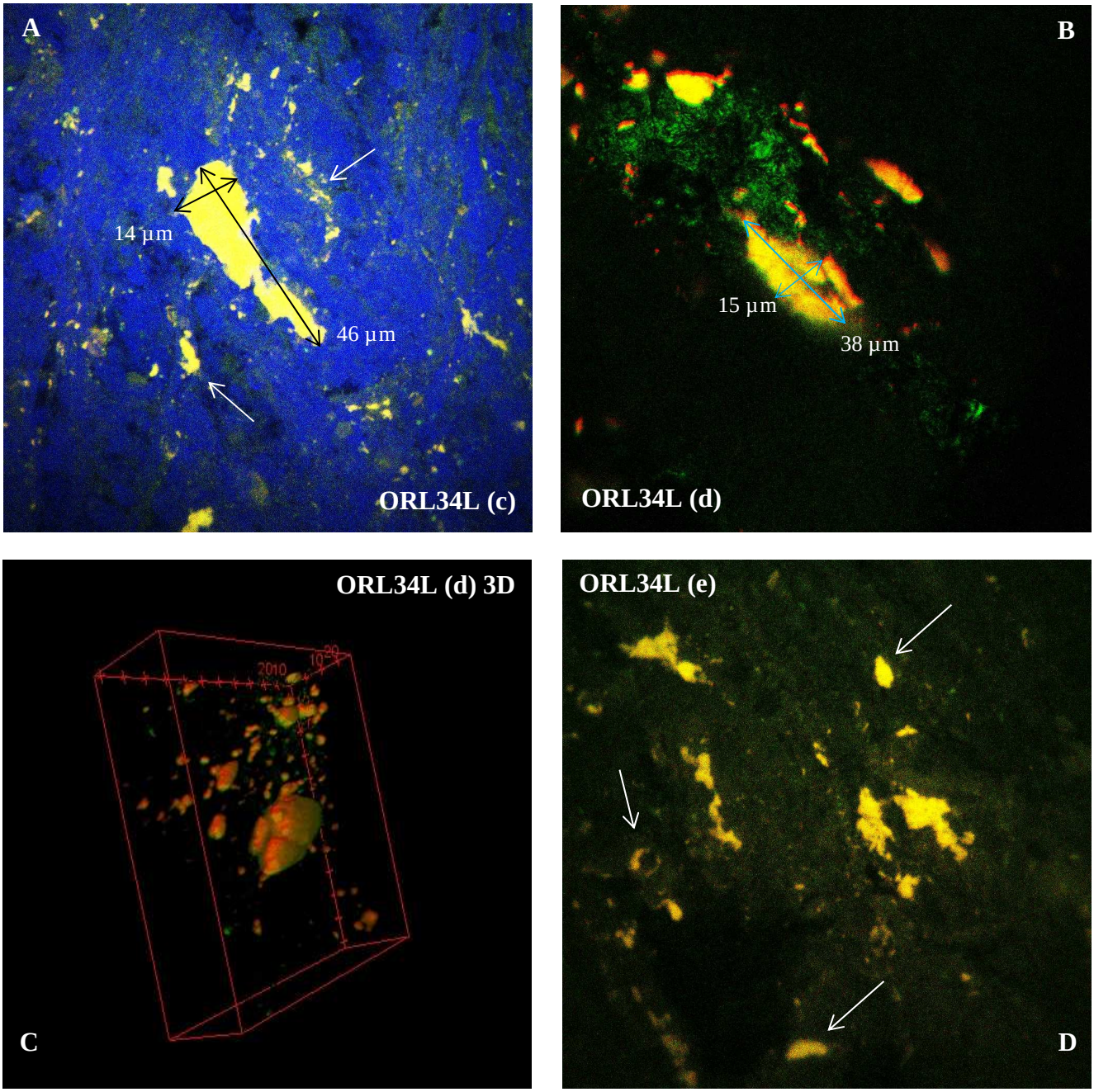

Figure 7: CLSM images of biofilm structures visualized by FISH. **(A)** A large biofilm structure of *H. influenzae* bacteria of 46x14 µm with planktonic – free floating bacteria around the biofilm structure (arrows). **(B)** A part of a biofilm structure of *H. influenzae* bacteria of 34 µm. Green staining indicates other bacterial involvement. This biofilm was reconstructed in 3D in **(C)**. **(D)** A biofilm structure of *H. influenzae* bacteria with planktonic – free floating bacteria (arrows) nearby the biofilm structure.

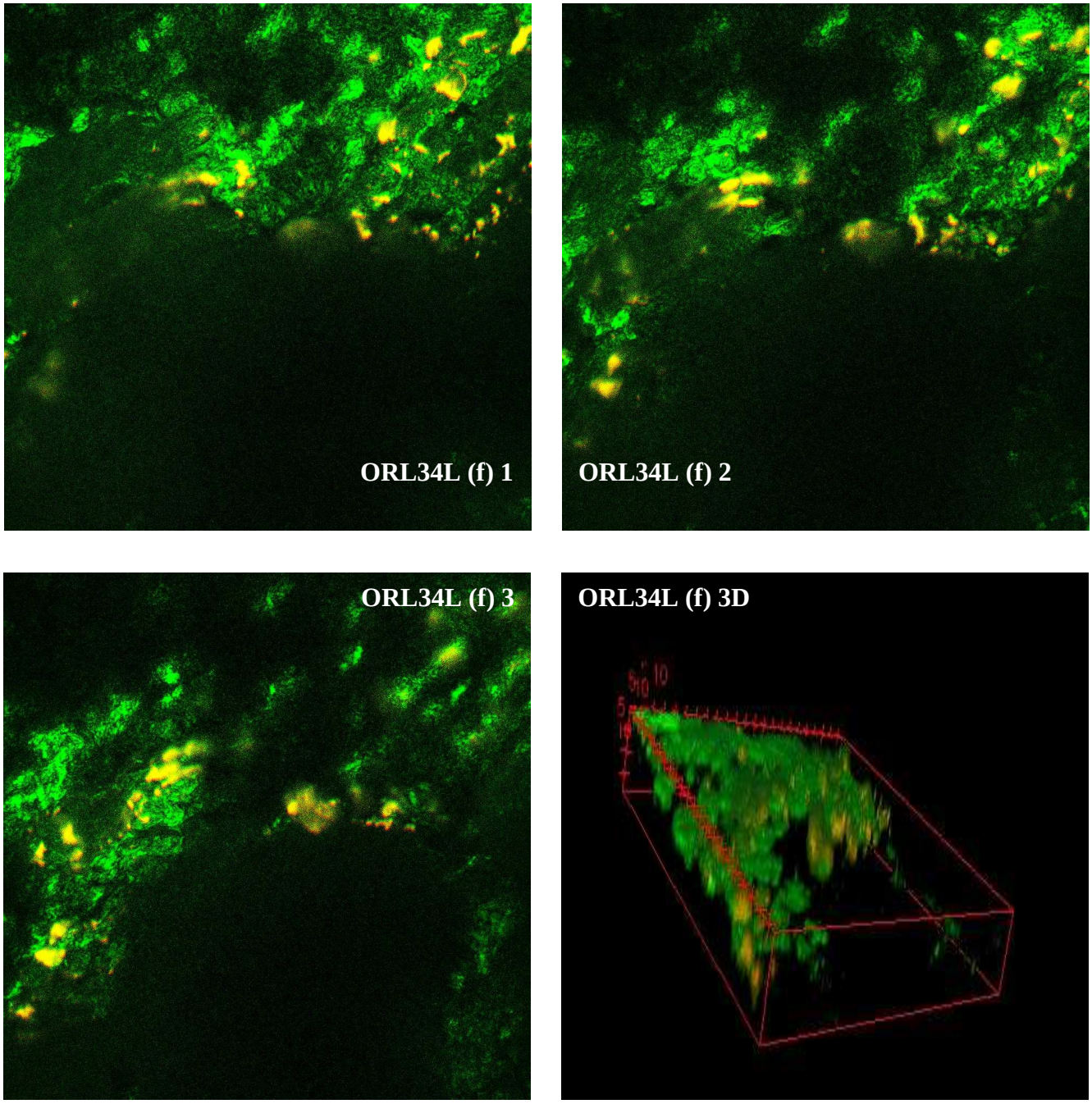

Figure 8: CLSM images of the same biofilm structure on different levels on the Z axis, visualized by FISH (1 highest, 3 lowest). Green staining indicates a polymicrobial biofilm structure. Yellow spots point to involvement of *H. influenzae* in this biofilm. A 3D view visualises the extensiveness of this polymicrobial biofilm structure in space.

Discussion

Although otitis media with effusion (OME) is a highly prevalent disease in children, the pathogenesis is still not well understood. OME does not respond well to antibiotics, which led to the hypothesis that biofilm structures are involved in the pathogenesis of this disease.

Biofilms have been demonstrated on the surface of the adenoid tissue, so it is assumed that the adenoid might act as a reservoir for otopathogenic bacteria. About the formation of biofilms in the middle ear of children with OME, there is only limited data available (25). Research groups have reported findings of biofilms in the middle ear mucosa of children (23, 25). Until this day, only one study (3) has been published in which biofilms were found in the middle ear effusions *in vivo.*

Trying to establish the role of biofilms in the pathogenesis of OME, this research focused on two main aspects. The first goal was to confirm the hypothesis that the adenoid may act as a reservoir for otopathogenic bacteria by identifying the same bacterial species and genotype in both the MEE and the adenoid. The second goal was to find evidence of the presence of biofilm structures in the middle ear effusions of children with OME. Therefore, qPCR and culture results were compared, which made it possible to find evidence of the so called 'biofilm paradigm'. This paradigm states that, because middle ear effusions of patients with OME are often culture negative, but bacteria can be found using qPCR, biofilm structures must be present. The second goal was to demonstrate the presence of biofilms *in vivo* by performing FISH and CLSM.

In this study, *Streptococcus pneumoniae* was the most frequently cultured bacterial species in the adenoid. It was found in 68% of all samples. *Haemophilus influenzae* was present in 59% of all samples. Dhooge, *et al*. (10) cultured *S. pneumoniae* only in 23% of the nasopharynx samples of patients. Instead, *H. influenzae* was found in 74% of samples and was therefore the most frequently found bacterial species. De Baere, *et al*. (24) cultured both *H. influenzae* and *S. pneumoniae* in the same quantity in the nasopharynx (40% of samples were positive).

Of all MEE samples in this study, 72% were culture positive for bacteria. This number is significantly higher than the 45.2% culture positive MEE samples Daniel, *et al.* (3) reported. (Z-test for proportion: $p<0.05$). Of all samples, 51% were culture positive for *H. influenzae*, *S. pneumoniae*, *Moraxella catarrhalis* or *S. aureus*. This is a significantly higher result than

Hall-Stoodley, *et al.* (23), who reported that only 19% of MEE samples were culture positive for *H. influenzae*, *S. pneumoniae* or *M. catarrhalis* (Z-test for proportion: $p<0.05$). Since in this study only 3 MEE samples were culture positive solely for *Staphylococcus aureus*, the fact that we included *S. aureus* in this comparison cannot explain this significant difference.

H. influenzae was present in 41% of the MEE samples. It was therefore the most frequently found bacterial species in the MEE samples, and can thus be regarded as the most important otopathogenic bacterial species in the pathogenesis of OME in this study. These findings only correspond to those of Thornton, *et al*. (25), who reported *H. influenzae* in 45% of samples. However, the samples used by Thornton, *et al*. (25) were middle ear mucosal biopsies, so one should be cautious when comparing these two. Other research groups reported significantly different results. Daniel, *et al.* (3) reported coagulase negative *Staphylococcus* (CoNS), *Veilonella* species and *S. aureus* to be the most prevalent in MEE samples. *S. pneumoniae* and *M. catarrhalis* were more prevalent than *H. influenzae*, which was present in only 3.2% of the MEE samples (3). This difference in prevalence of *H. influenzae* proved to be significant (Z test for proportion: $p<0.05$). Hall-Stoodley, *et al.* (23) also reported *H. influenzae* to be the most frequent otopathogenic bacterial species found by culturing, but only 7% of the MEE samples were culture positive for *H. influenzae* in their study. This percentage is significantly lower than our findings (Z-test for proportion: $p<0.05$). However, Hall-Stoodley, *et al.* (23) also reported that *H. influenzae* was found in 71% of samples by PCR, which is significantly higher than our results (Z-test for proportion: $p<0.05$) (23).

Possible explanations for the significant differences in bacteriological findings between this study and previously reported literature are differences in population (age, in- and exclusion criteria) and methodology. Dhooge, *et al.* (10) and De Baere, *et al.* (24) used the same exclusion criteria as those used in this study, but Dhooge, *et al.* (10) studied children with recurrent acute otitis media, De Baere, *et al. (24)* included patients scheduled for middle ear surgery, but not specifically with the diagnosis of OME. The culture media used by Dhooge, *et al.* (10), De Baere, *et al*. (24), Hall-Stoodley, *et al.* (22) and Daniel, *et al*. (3) differed from those used in this study. (See addendum III – table IV) Hall-Stoodley, *et al.* (22) only cultured in an aerobic environment at 5% CO_2, but not in an anaerobic environment. In this study, both aerobic and anaerobic culturing was performed. Daniel, *et al.* (3) also performed aerobic and anaerobic culturing, but anaerobic culturing was only performed with sheep blood agar, which is not suited to grow *H. influenzae.*

De Baere, *et al.* (24), Hall-Stoodley, *et al*. (22) and Daniel, *et al*. (3) all used a different study population, which was older than the population studied in this research. (See addendum III – table IV). Usage of antibiotics in the month prior to this study was an exclusion criterion in this study, while Hall-Stoodley, *et al.* (22) did not exclude patients who used antibiotics. These parameters have to be taken into account when comparing results from different studies.

In order to support the hypothesis that the adenoid acts as a potential reservoir for otopathogenic bacteria, results from culturing and genotyping were analysed. In 19/34 patients (55.9%), the same bacterial species was found in the MEE and in the adenoid. This percentage is significantly higher than the percentage found by Emaneini, *et al*. (22), who found the same bacterial species in 31% of the studied population (Z-test for proportion: $p<0.05$). The same bacterial genotype was found in the middle ear and the adenoid in 12/34 patients (35%) included in this study. This percentage is higher, but not significantly different from the percentage found by Emaneini, *et al*. (Z-test for proportion: $p>0.05$), who reported to have found a relation in genotype in 29% of patients (22). It should be noted that in the study of Emaneini, *et al*. a different method for genotyping was used (pulsed field gel electrophoresis) than in this study (McRAPD) (22).

These findings, together with the fact that a statistical significant correlation could be found between the presence of the same bacterial species in the adenoid and the MEE on one hand and the presence of the same bacterial genotype on these places on the other hand, point in the direction that the adenoid indeed acts as a reservoir for otopathogenic bacteria, which was one of the goals to investigate of this study.

Previous research has shown that MEE and adenoid samples often had negative culture results, but positive PCR results (24). Hall-Stoodley, *et al.* (23) reported that only 19% of MEE samples were culture-positive for *H. influenzae, S. pneumoniae* or *M. catarrhalis*, while 100% of the MEE samples assessed by PCR were positive for at least one of these three bacteria. De Baere, *et al.* (24) reported that 55% of MEE samples were culture negative, but CLSM proved the presence of bacteria in all MEE samples. These findings have led to the hypothesis that bacteria present in the middle ear reside in a biofilm formation, the so called biofilm paradigm. This paradigm suggests that bacteria in biofilm are often difficult to culture, but their presence can be demonstrated qPCR. So, when biofilms are present, one may expect a high number of culture negative but qPCR positive samples.

In this study, 23.6% of all samples (MEE, adenoid and nasopharynx samples combined) had a culture negative but qPCR positive result. Although the fact that this result proved to be not significant, this percentage might suggest the presence of biofilms in an important number of cases. These results are lower than those reported in literature (23, 24). This can be explained by the fact that the combination of culture and qPCR was performed on a very low number of patients in this study. (N=7 for MEE samples)

Biofilm formation on adenoid tissue has been previously reported in literature (21). Therefore, the focus of this study was to investigate biofilm formation in MEE. In this study, 21.4% of the MEE samples had a culture negative but a qPCR positive result.

In 15.5% of all 110 cases in which qPCR and culture results of MEE, adenoid and nasopharynx samples were compared, a culture positive, but qPCR negative result was found. Since qPCR is a more sensitive technique than culturing, this is counterintuitive. Possible reasons for this result can be found in potential contamination of culture plates, problems with standard series used in qPCR or human errors in performing qPCR. These topics are further discussed below (see: *3. Pitfalls*).

Using CLSM, live bacteria were seen in 100% of the investigated MEE samples. This percentage is higher, but not significantly different from the findings of Daniel, *et al.* (3), who reported to have found live bacteria in 82.3% of MEE samples. The population studied by Daniel, *et al*. (3) was older than the population studied in this research, but they reported to have found more live bacteria in MEE samples of children than of adults with CLSM, which supports our findings.
H. influenzae specific biofilm structures were detected in 5 out of 10 studied samples (50%) with CLSM. This percentage matches the percentage of samples in which biofilm structures were detected by Daniel, *et al.* (3). However, Daniel, *et al.* (3) detected biofilms of different bacterial species, while in this study only *H. influenzae* specific biofilms were studied, which may have biased the results.

Pitfalls

A. Study population

In this relatively small study of 34 patients, rigorous inclusion and exclusion criteria were used. When comparing the results with the literature, care must be taken to compare the different populations studied as age and other in- and exclusion criteria can influence the results.

B. Nasopharynx versus Adenoid samples

Based on the literature, nasopharynx swabs are considered to represent the adenoid microflora. This is supported by the results of this study. However, some differences between culture results of nasopharynx and adenoid samples were noted. A possible explanation for these different culture results is the fact that nasopharynx samples were collected using a swab which was brought into the nasopharynx through the nose, where the swab was possibly contaminated with bacteria present in the nose, but not in the nasopharynx or the adenoid. Another explanation for these differences is that some bacterial species are present in the crypts of the adenoid, but are not present at the surface of this tissue. Nasopharynx swabs may thus represent the bacterial microflora present on the surface of the adenoid tissue, but miss the bacteria present in the crypts of the adenoid. This might have interfered with the results of this study.

C. Culture techniques

Samples of MEE and adenoid tissue were cultured only on CHOC plates, which has led to a narrow culture approach. This is in contrast with other studies, which used more and different culture media. (See addendum III – table IV) This narrow culture approach might have led to the fact that specific bacterial species, which were better adapted to growing on CHOC plates, were selected or were able to overgrow other bacteria present in the samples. It is possible that this plays a role in the observation that *H. influenzae* was the most frequently found bacterial species in MEE samples, which is not supported by other research groups. However, it is known that *H. influenzae* will most likely not overgrow other bacteria. The finding of high quantities of *H. influenzae* thus confirms the use of good culture conditions. On the other hand, it is also possible that the presence of *H. influenzae* was underestimated by other research groups because of the fact they did not use *H. influenzae* selective culture media (See addendum III – Table IV).

Culturing bacteria on culture plates is a manual technique which often requires some finesse and experience. When different bacterial species are present on a culture plate, they have to be detected on sight and re-isolated by hand. This fact makes it possible that bacteria, present in low numbers in the samples or not able to grow well on CHOC plates, formed few and/or small colonies, which were then overlooked. This leads to the possibility that different bacterial species, present in the samples, were not recognised and thus missed.

D. Identification

To identify bacteria, MALDI-TOF/MS was used. Although the many benefits of this technique (lower workload with respect to t-DNA identification, fast identifications), it must be stated that a few limitations were encountered. First of all, the bacterial colonies had to be of good quality, could not be too old or too dehydrated and had to be pure for identifications.

Secondly, problems can occur with the matrix used to cover the spots. This matrix evaporates very quickly, which makes it possible that the composition of this matrix was not always good, which may have interacted with the reliability of the identifications.

Another pitfall in the use of MALDI-TOF/MS is the fact that the identification of bacteria is software dependent. If the used library is not up to date, this can lead to unreliable identifications. In the course of using the MALDI-TOF/MS machine, it also becomes polluted, which may interact with the quality of the identifications.

E. Genotyping

McRAPD was used in this study to genotype identified bacteria. This technique implies that melting curves have to be analysed. This analysis is sometimes subjective and requires some experience. However, when results were vague and difficult to interpret, the test was repeated or gel electrophoresis was performed, which is more time consuming but easier to interpret.

F. Quantative PCR (qPCR)

Quantification of bacteria was performed by qPCR. To interpret the results of qPCR, experience is important. Because of the fact that qPCR results are measured relatively to a standard series, which was pre-produced by a laboratory staff member, problems with the standard series may have influenced the qPCR results.

Because of the fact that the sample volume was too small to perform both qPCR and FISH on the same sample, qPCR was only performed on samples from patients included in the pilot

study. In this pilot study, a few MEE samples were used to optimise the FISH protocol for the final study. This is why qPCR on MEE samples was performed on only 7 patients, which makes it difficult to extrapolate the findings from the qPCR results to a larger population.

The comparison between culture and qPCR results led to the finding that 15.5% of all cases (MEE, adenoid and nasopharynx) had culture positive but qPCR negative results. This, in combination with the relatively high number of qPCR negative results, can be explained by the possibility that blood, present in the MEE samples, led to inhibition of the PCR reaction and thus led to a false negative result.

G. FISH and CLSM

Different problems were encountered when trying to perform FISH and CLSM on the MEE samples of our patients. First of all, the research was delayed because of the use of bad manufactured probes. This led to consecutive (false) negative results. Another problem that had to be dealt with was the specific viscous consistency of the MEE samples, which made it hard to bring the entire sample on a sample slide and to fixate the specimen. The presence of red blood cells in the MEE samples made it impossible to perform CLSM at first. Later, a lysis buffer was used to lyse these cells, but not only red blood cells, but other cells in the specimen too were affected, which also led to unreliable results. Finally, another lysis buffer was used (acetic acid), which made it possible to get reliable FISH and CLSM results. However, some samples still contained too much blood to be able to perform CLSM.

Little is known in literature about the best way to preserve MEE samples, to keep the bacteria in the samples alive and retain possible biofilm structures present in the samples at the same time. Because of this fact, there was no certainty about how MEE and adenoid samples from patients included in this study had to be stored. Thereby, the samples were first stored at -80 °C, but were later moved to a -20 °C storage room when fear arose that biofilm structures would be harmed by the low temperatures. Some samples (the most recent ones) were stored only at -20 °C. These differences in cryopreservation temperatures may have led to differences in the presence of biofilm structures between samples.

Another pitfall that has to be mentioned is the fact that the interpretation of FISH and CLSM results is often subjective and not very reproducible. Daniel, *et al*. state that biofilm is present when three-dimensional bacterial clusters within an amorphous matrix are present, associated with a surface such as eukaryotic cells or strands. Hall-Stoodley, *et al*. say biofilms are

present when pathogenic bacteria can be found in clusters within a matrix attached to a surface (3, 23). In practice, it is difficult to decide objectively whether bacteria form a cluster and thus reside in a biofilm formation, or are just located near each other by chance. The difference between free bacteria, not comprised in a biofilm formation, and biofilm bacteria in a planktonic phase is difficult to determine.

Another fact that can impede the finding of biofilms in the MEE samples is that biofilms can be present in only a part of the effusion. Only when the entire MEE sample is analysed, it can be stated with certainty that biofilms are present or not. Since a part of each MEE sample had already been used for culturing, it is possible that biofilms were present, but were not detected with FISH or CLSM.

Since *H. influenzae* was the most frequently found bacterial species in the MEE samples and because of the complexity of the technique and the high price of species specific probes, only *H. influenzae* specific probes were used. However, *S. pneumoniae, M. catarrhalis* and *S. aureus* are known to form biofilms too. The fact that these species were not visualised, makes it possible that some biofilm structures present in the samples were not detected.

Treatment options

OME is a disease with a high percentage of natural resolution. Therefore, in uncomplicated cases of OME, watchful waiting is considered to be the standard care. No medical treatment has a long term beneficial effect on the resolution of OME. When effusions persist for more than 3 months, surgical treatment can be considered.

The ineffectiveness of antibiotics has been discussed in different studies (1). This finding is in agreement with the possible role of biofilm in OME. Biofilm bacteria are difficult to culture and are recalcitrant to antibiotic treatment for indolent long-term persistence.

Surgical treatment consists of tympanostomy tube insertion and adenoidectomy. The role of adenoidectomy can be supported by our study, because it functions as a reservoir for otopathogenic bacteria. The insertion of the tympanostomy tubes is performed to clear the MEE, which might contain biofilms. One of the main problems of this treatment is that, after 6-9months, the tubes will be expulsed, but 20-25% of the children require new placement of tubes within 2 years (41).

Since 80% or more of the children experience at least one episode of OME, it is important to find alternative treatments. Daniel, *et al.* suggested novel antibacterial strategies such as locally delivered high-dose antibiotics over a prolonged period of time or new drug delivery systems and antimicrobial impregnated devices, which appear promising. Other novel techniques such as ultrasound low-strength electrical fields, enzymatic degradation of extracellular matrix, inhibition of quorum sensing, disruption of biofilm-related genes, or a combination of the above in a smart system that detects and treats biofilm infections, might be of interest (3). Daniel and Chessman investigated the use of antibiotic pellets in middle ear. The pellets with medium or high dose of antibiotics eradicated the biofilm successfully in vitro. It supports the idea that local treatment is much more effective than systemic antibiotics (41).

Conclusion

The results of this study support the hypothesis that the adenoid indeed acts as a reservoir for otopathogenic bacteria and thereby facilitates infection of the middle ear. Secondly, the findings in this study indicate that biofilms, specifically consisting of *H. influenzae*, are present in the middle ear effusions of children with COME. This leads to the conclusion that biofilms may play a crucial role in the pathogenesis of otitis media with effusion, which is important in the understanding of this disease and the development of potential future treatment options.

On the other hand, this study shows that finding proof of biofilms in MEE *in vivo* is not obvious and is associated with a large number of problems and technical difficulties. Further research to optimise this technique and find more proof of biofilms present in the MEE is therefore needed.

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Addendum I

Table I: *Culture results*			
	Middle ear	**Adenoid**	**Nasofarynx**
Species			
Abiotrophia defectiva	0	1	0
Acinetobacter baumannii	0	1	0
Actinomyces odontolyticus	0	**4**	0
Arcanobacterium pseudopyogenes	0	1	0
Arthrobacter sp.	0	1	0
Bacillus circulans	0	0	1
Bacillus licheniformis	1	0	0
Bacillus pseudomycoides	1	0	1
Bacillus subtilis	0	1	0
Bordetella bronchiseptica	0	0	1
Corynebacterium argentoratense	0	1	0
Corynebacterium propinquum	0	1	1
Cornynebacterium pseudodiphtheriticum	0	0	**7**
Eikenella corrodens	0	3	0
Enterococcus faecalis	0	1	0
Enterococcus gallinarum	0	1	0
Fusobacterium necrophorum	0	2	0
Gemella haemolysans	0	0	1
Granulicatella adiacens	0	**4**	1
Granulicatella elegans	0	1	0
Haemophilus influenzae	**8**	**7**	**6**
Inquilinus limosus	0	1	0
Lactobacillus paracasei	1	1	0
Lactobacillus species	0	0	1
Moraxella catarrhalis	0	**4**	**3**
Neisseria sp.	0	1	0
Neisseria meningitidis	0	1	0
Neisseria flavescens	0	1	0
Paenibacillus sp.	1	0	0
Parvimonas micra	0	1	0
Prevotella buccae	0	1	0
Propionibacterium acnes	0	2	2
Propionibacterium granulosum	0	1	0
Pseudomonas aeruginosa	0	1	2
Pseudomonas pseudoalcaligenes	0	1	0
Pseudomonas stutzeri	1	1	0
Rothia mucilaginosa	0	3	0
Salmonella sp.	0	0	1

Staphylococcus aureus	**3**	**6**	**5**
Staphylococcus capititis	2	1	1
Staphylococcus caprae	**3**	0	0
Staphylococcus epidermidis	**4**	3	**6**
Staphylococcus hominis	2	0	0
Staphylococcus rostri	0	1	0
Staphylococcus species	1	0	0
Staphylococcus succinus	0	0	1
Streptococcus anginosus	0	3	1
Streptococcus constellatus	0	1	0
Streptococcus cristatus	0	1	0
Streptococcus hyointestinalis	1	0	0
Streptococcus mitis	0	2	0
Streptococcus parasanguinis	0	2	1
Streptococcus pneumoniae	**3**	**9**	2
Streptococcus pyogenes	0	1	1
Streptococcus salivarius	0	2	0
Tetrathiobacter kashmirensis	0	1	0
Turicella otitidis	1	0	0
Veillonella parvula	0	1	0
Veilonella species	0	1	0

Legend: *Each number relates to the number of isolates found in the samples from all patients for each bacterial species. The most frequently found species were marked in bold.*

Table II: *Number of bacterial species cultured per patient*				
	Right ear	Left ear	Adenoid	Nasopharynx
ORL 09	N	1	9	N
ORL 10	3	N	8	6
ORL 11	3	4	6	6
ORL 13	2	1	6	3
ORL 14	5	1	2	6
ORL 15	1	1	2	6
ORL 16	4	2	5	6
ORL 17	1	N	12	4
ORL 18	0	0	5	3
ORL 19	1	0	5	1
ORL 20	1	1	4	3
ORL 21	2	1	9	4
ORL 22	1	0	9	5
ORL 23	3	1	11	
ORL 24	1	2	9	
ORL 25	2	0	8	
ORL 26	2	0	11	
ORL 27	0	0	7	
ORL 28	3	2	5	
ORL 29	0	4	8	
ORL 30	0	3	13	
ORL 31	1	N	9	
ORL 32	N	1	12	
ORL 33	3	N	8	
ORL 34	1	1	6	
ORL 35	N	0	9	
ORL 36	1	0	3	
ORL 37	0	1	6	
ORL 38	N	0	4	
ORL 39	N	0	3	
ORL 40	N	3	7	
ORL 41	0	2	5	
ORL 42	N	4	9	
ORL 43	N	2	3	

Legend: *N*: *No sample,* ***Shaded***: *Samples not collected in final study.*

Table III: *Bacterial load according to qPCR (bacteria/ml)*				
Sample	***H. influenzae***	***S. pneumoniae***	***M. catarrhalis***	***S. aureus***
ORL09 MEE Re	N	N	N	N
ORL09 MEE Le	0	0	0	8.48E3
ORL09 Adenoid	2.38E4	0	0	0
ORL09 Naso	N	N	N	N
ORL10 MEE Re	0	/	0	/
ORL10 MEE Le	N	N	N	N
ORL10 Adenoid	/	/	/	/
ORL10 Naso	N	N	N	N
ORL11 MEE Re	4.12E4	0	0	1.11E4
ORL11 MEE Le	2.15E5	0	0	0
ORL11 Adenoid	8.24E6	2.18E4	0	0
ORL11Naso	1.30E5	5.85E5	2.07E7	3.85E2
ORL12 MEE Re	8.69E2	0	0	1.50E4
ORL12 MEE Le	N	N	N	N
ORL12 Adenoid	/	/	/	/
ORL12 Naso	N	N	N	N
ORL13 MEE Re	/	/	/	/
ORL13 MEE Le	/	/	/	/
ORL13 Adenoid	8.47E4	3.19E4	0	0
ORL13 Naso	N	N	N	N
ORL14 MEE Re	9.30E4	1.14E2	0	0
ORL14 MEE Le	1.10E5	1.08E3	0	0
ORL14 Adenoid	1.13E6	4.25E4	0	0
ORL14 Naso	6.75E4	2.96E6	2.14E6	1.11E2
ORL15 MEE Re	0	0	0	0
ORL15 MEE Le	0	0	0	0
ORL15 Adenoid	1.23E4	7.88E5	0	0
ORL15 Naso	1.60E5	3.88E6	0	8.85E3
ORL16 MEE Re	7.05E2	1.44E2	0	3.34E2
ORL16 MEE Le	3.97E3	0	0	Postive (bad quant.)
ORL16 Adenoid	2.29E4	5.2E4	0	0
ORL16 Naso	2.93E6	1.08E7	0	3.84E4
ORL17 MEE Re	0	0	0	6.65E3
ORL17 MEE Le	N	N	N	N
ORL17 Adenoid	1.23E4	0	0	0
ORL17 Naso	3.30E2	1.44E3	0	4.74E3
ORL18 MEE Re	/	/	/	/
ORL18 MEE Le	/	/	/	/
ORL18 Adenoid	0	0	0	0
ORL19 MEE Re	/	/	/	/
ORL19MEF Le	/	/	/	/
ORL19 Adenoid	1.23E4	0	0	0
ORL19 Naso	/	/	/	/
ORL20 MEE Re	/	/	/	/

ORL20 MEE Le	/	/	/	/
ORL20 Adenoid	5.42E4	0	0	0
ORL20 Naso	/	/	/	/
ORL21 MEE Re	/	/	/	/
ORL21 MEE Le	/	/	/	/
ORL21 Adenoid	2.28E5	8.53E5	0	0
ORL21 Naso	/	/	/	/
ORL22 MEE Re	/	/	/	/
ORL22 MEE Le	/	/	/	/
ORL22 Adenoid	1.16E6	0	0	0
ORL22 Naso	/	/	/	/

Legend:*N*: *No sample,* ***/***: *No test performed,* ***0***: *Negative result.*

Addendum II

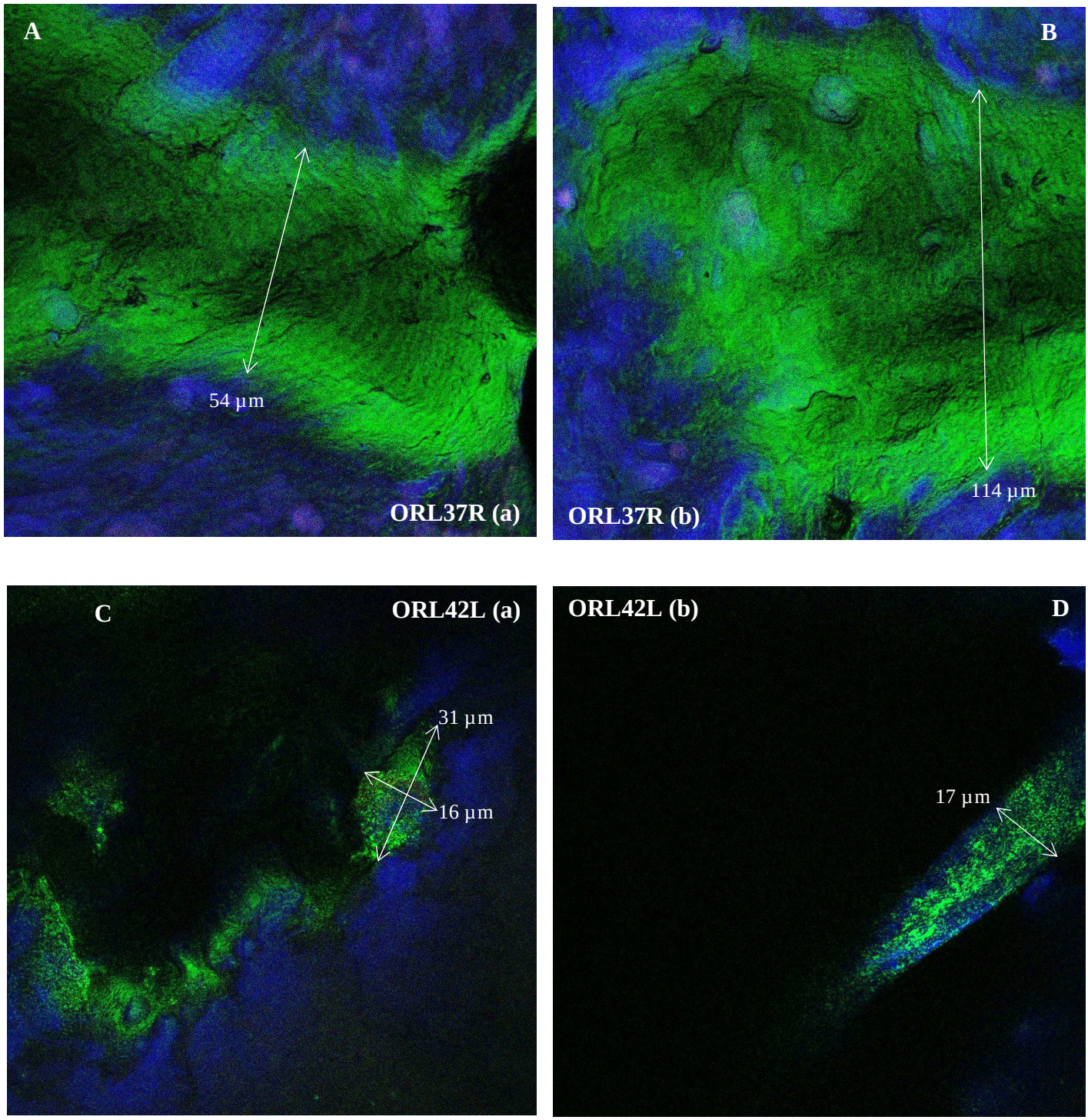

***Figure I*:** CLSM images of bacterial clusters. **(A&B)** No *H. influenzae* was detected in this culture negative sample, which acted as a negative control. The green staining suggests that biofilm structures are present in this sample, which do not contain *H. influenzae*, however the distinction with background noise is hard to make. **(C&D)** No *H. influenzae* was detected in this culture positive sample. However, nonspecific bacterial clusters were detected.

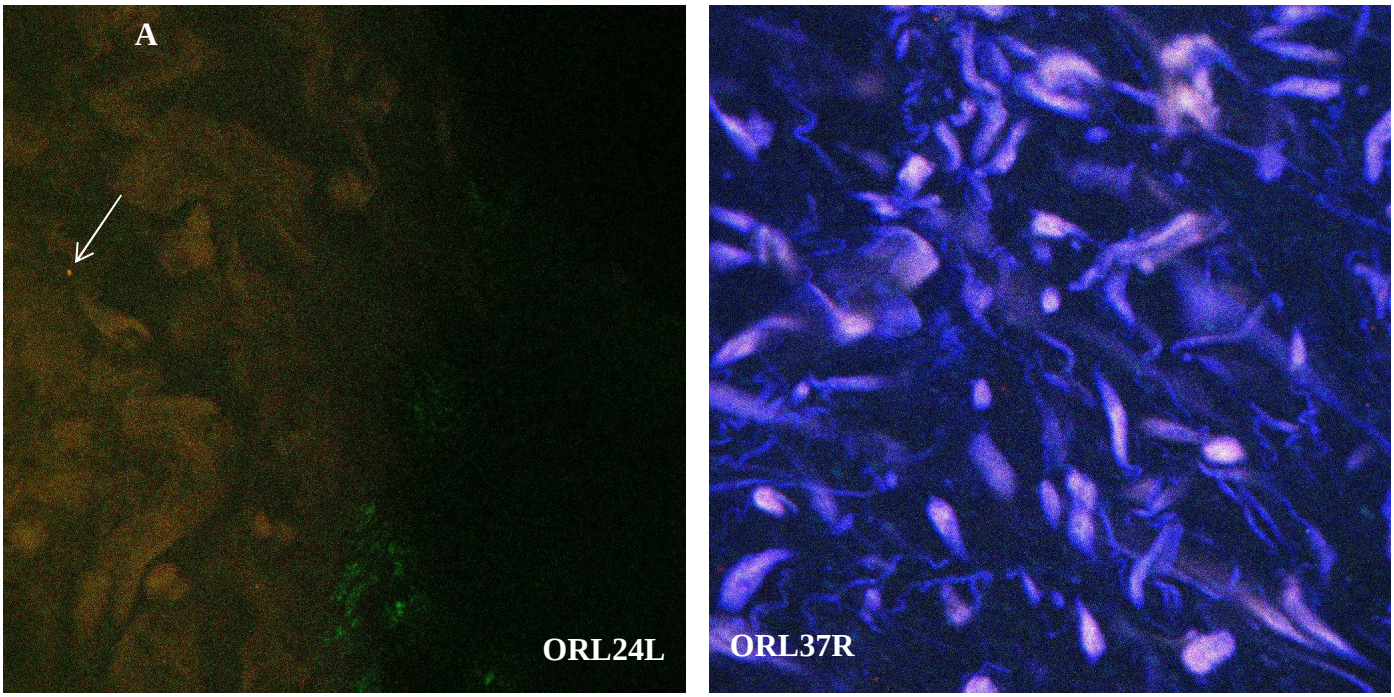

***Figure II*:** CLSM images of MEE samples. **(A)** Background noise dominates this image. The distinction between bacterial clusters and background noise is hard to make. One single *H. influenzae* bacteria was detected (arrow). **(B)** Uncommon shapes suggest artefacts in this part of the sample.

Addendum III

Table IV: *Summary and characteristics of key articles for this study.*

Key article	Author	Publication year	Number of patients included	Culture techniques	Population age
Role of nasopharyngeal bacterial flora in the evaluation of recurrent middle ear infections in children	Dhooge, *et al.*	1999	35	GC-chocolate agar, Tryptic Soy agar + 5% sheep blood, selective agar for *M. catarrhalis* (aerobic and anaerobic)	6 months – 4 years old
Otitis media microbes: culture, PCR, and confocal laser scanning microscopy	De Baere, *et al.*	2009	14	Tryptic soy agar + 5% sheep blood, Gonococcal Culture II agar (aerobic and anaerobic)	5 years – 55 years old
Bacterial involvement in otitis media with effusion	Daniel, *et al.*	2012	42	Sheep blood agar , MacConkey agar, chocolatised blood agar for *H. influenzae*, *H. pylori* and Mycoplasma selective agars (aerobic) and sheep blood agar (anaerobic).	1 year – 75 years old
Direct detection of bacterial biofilms on the middle-ear mucosa of children with COME	Hall-Stoodley, *et al.*	2006	26	Blood agar, Chocolate agar, MacConkey agar and colistin nalidixic acidblood agar (only aerobic)	6 months – 15 years old
Multi-species bacterial biofilm and intracellular infection in otitis media	Thornton, *et al.*	2011	20	Blood agar, cysteine lactose elektroylyte deficient agar, Filde's agar, colistin nalidixic acid blood agar (aerobic) and blood agar and colistin nalidixic acid blood agar (anaerobic)	0 – 10 years old
The biofilm paradigm as the elucidation of otitis media with effusion	De Paepe, Lambert	2014	34	CHOC	1 – 6 years old

Addendum IV

Side project: Helicobacter pylori

During this study, we stumbled upon conspicuous literature suggesting a possible role of Helicobacter pylori in the pathogenesis of OME. Recent studies showed the presence of *H. pylori* in MEE of patients with OME suggesting that it could play a role in the etiology of OME (1, 2, 3). *H. pylori* is a gram negative bacterial species that colonizes the stomach of half of the world's population. This species is associated with an increased risk of noncardia gastric adenocarcinoma, gastric lymphoma and peptic ulcer. On the other hand, chronic colonization of the stomach with *H. pylori* reduces the risk of reflux (4, 5).

In this side project, the presence of *H. pylori* in OME was investigated. Fifty-seven MEE samples, 34 adenoid and 11 nasopharynx samples were cultured. PCR was performed on a part of these samples (28 MEE samples, 12 adenoid samples and 4 nasopharynx samples).

H. pylori was cultured in 0/57 MEE samples, 0/34 adenoid samples and in 0/11 nasopharynx samples. Subsequently PCR was performed, of which the products were analysed with gel electrophoresis. The results of the gel electrophoresis of MEE and nasopharynx are shown in Figure I. All results were negative, with row 1 and 2 being the positive control and row 35 and 36 the negative control. The results of the gel electrophoresis of 12 adenoid samples are shown in Figure II. These tests were negative as well with row 15 being the positive control.

The findings in this study differ to a great extent of what other publications suggest. Yilmaz et al. (2005) (2) reported that in a group of 22 children with OME, 16/34 (47%) MEE samples were positive for *H. pylori*. Melake et al (3) showed evidence of the presence of H. pylori in 56% of MEE samples of 60 children with OME.
A possible explanation for these findings is the fact that *H. pylori* is more frequently found in Eastern than in Western populations. Another explanation is the hypothesis that *H. pylori* reduces the risk of reflux by diminishing the gastric acidity (4, 5).
We can conclude that our findings do not support the hypothesis that *H. pylori* is involved in the pathogenesis of OME, as stated in literature.

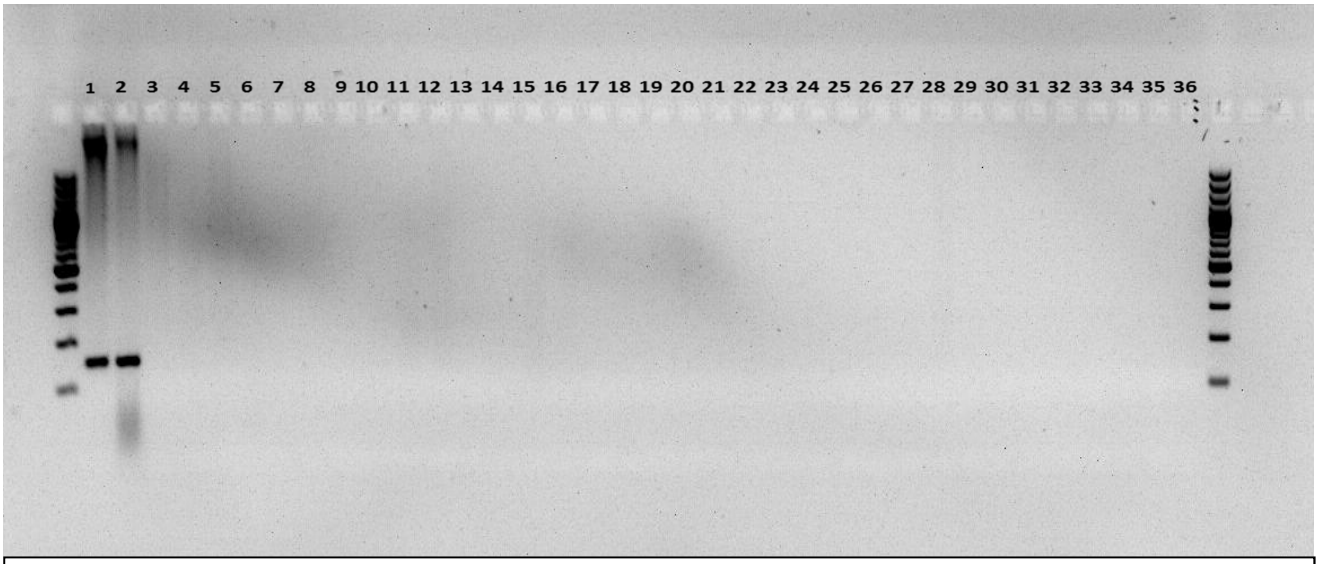

Figure I: *Visualisation of PCR products of MEF and nasopharynx after gel electrophoresis on 1% agarose gel with 1 and 2 as positive control.*

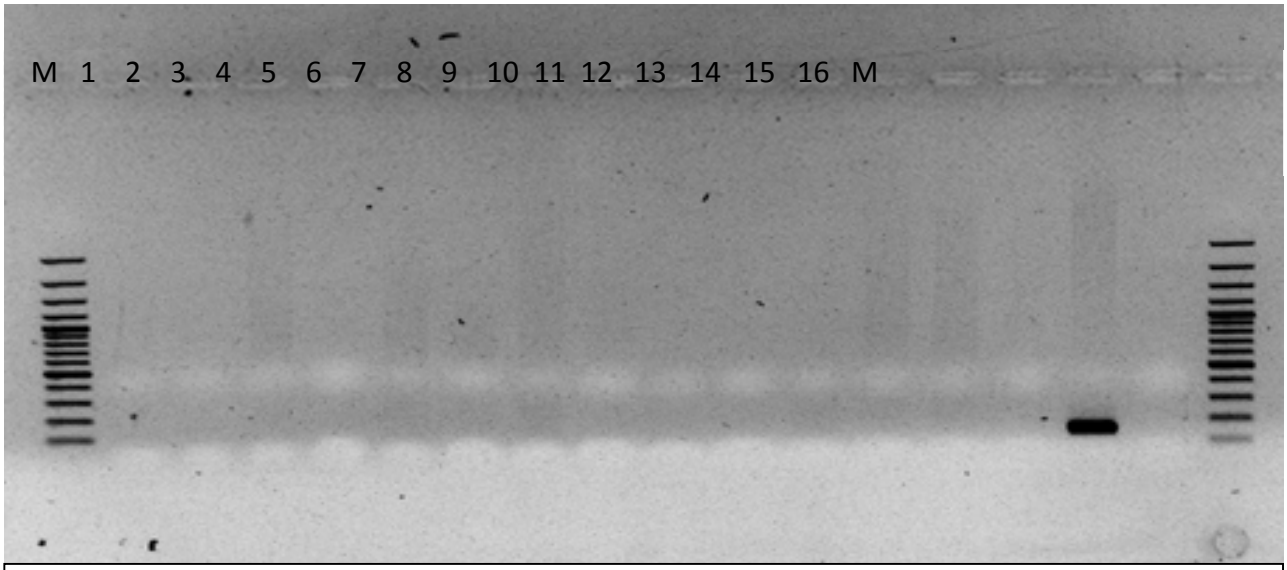

Figure II: *Visualisation of PCR products of adenoid samples after gel electrophoresis on 1% agarose gel with 15 as positive control.*

References side project

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