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OTOLOGY

Haemophilus influenzae biofilm formation in chronic otitis media with effusion

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Abstract Otitis media with effusion (OME) is a highly prevalent disease in children, but the exact pathogenesis and role of bacteria are still not well understood. This study aimed to investigate the presence of otopathogenic bacteria in the middle ear effusion (MEE) and adenoid of children with chronic OME (COME), and to investigate in vivo whether these bacteria, especially *Haemophilus influenzae*, are organized as a biofilm in the middle ear fluid. MEE and adenoid samples were collected from 21 patients with COME. Extensive bacterial culturing and genotyping was performed on all middle ear and adenoid samples. Fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) was used to visualize possible biofilm structures for a selection of middle ear effusion samples. 34 MEE samples were collected from 21 patients of which 64.7 % were culture positive for bacteria and 47.0 % were culture positive for *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and/or *Streptococcus pneumoniae*. All 21 adenoid samples were culture positive for one or more of these four otopathogens. *H. influenzae* (35.3 %) and *S. pneumoniae* (76.2 %) were the most frequently cultured bacteria in the MEE and adenoid samples, respectively. The same bacterial species was found in MEE and adenoid for 84.6 % of the patients and in 81.2 % of the cases where the same species was found in more than one site it involved the same bacterial genotype. FISH and CLSM demonstrated the presence of *H. influenzae* specific biofilm structures in five of the eight culture positive MEEs that were tested, but in none of the two culture negative MEEs. The findings in this study indicate that the adenoid acts as a reservoir for bacteria in MEE and confirms that biofilms, in at least half of the cases consisting of *H. influenzae*, are indeed present in the MEE of children with COME. Biofilms may thus play a crucial role in the pathogenesis of COME, which is important in the understanding of this disease and the development of potential future treatment options.

Keywords Chronic otitis media with effusion (COME) · Biofilm · Fluorescent in situ hybridization (FISH) · Confocal scanning laser microscopy (CLSM) · *Haemophilus influenzae*

Introduction

Otitis media with effusion (OME) is the most common inflammatory disease of the middle ear, characterized by the presence of fluid in the middle ear cavity, behind an intact tympanic membrane. Typically, there are no signs or symptoms of acute infection. OME mainly affects children at a critical age of speech and language development, with hearing loss as the major complaint.

OME is generally considered as a benign condition with a high percentage of spontaneous recovery [1–4]. However,

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if OME persists for more than 3 months, it is defined as chronic otitis media with effusion (COME). For COME associated with a prolonged and significant hearing loss and/or a negative impact on the child's educational and developmental status, behavior or social well-being, the guidelines of the National Institute for Health and Care Excellence (NICE) recommend the insertion of transtympanic ventilation tubes (TVT) or the fitting of hearing aids when surgery is contraindicated [5].

OME was previously thought of as a sterile inflammatory process, as bacterial cultures were frequently negative. Later, nucleotide amplification techniques demonstrated that these effusions contain genomic material of pathogenic bacteria, which remains present up to 4 weeks after treatment with antibiotics [6]. Furthermore, bacterial mRNA and proteins have also been found in the effusions, indicating that these bacteria remain metabolically active [7]. These findings lead to the hypothesis that, in OME, otopathogenic bacteria live in a specialised structure, called 'biofilm' [8].

Biofilms are robust communities of microbes encased in a self-produced hydrated matrix of polysaccharides and proteins, attaching and growing on synthetic or natural surfaces, including human tissue. Bacteria in biofilms organize into complex three-dimensional micro-colonies. The extracellular matrix of a biofilm structure protects its bacteria not only against hostile environmental conditions, e.g. changes in temperature, moisture and pH, but also against phagocytosis and humoral immunity [9]. Furthermore, bacteria in biofilms show increased antibiotic resistance due to several mechanisms, including the protective polysaccharide matrix, the decreased bacterial metabolic activity within the biofilm, the increased level of mutations in antibiotic target molecules and upregulated efflux pumps [10]. As formation of biofilms improves bacterial defensive skills, biofilms are a preferred mode of existence of many microbial species and are implicated in several chronic bacterial infections [11].

Biofilm formation in the middle ear was first demonstrated in chinchillas with experimentally induced chronic suppurative otitis media [12]. Later, evidence of biofilm presence in cholesteatoma and in mucosal biopsies of patients with chronic suppurative otitis media without cholesteatoma was shown by scanning electron microscopic imaging [13–15].

Only few studies have studied biofilms in otitis media with effusion. Hall Stoodley et al. [11] were the first to demonstrate biofilms on middle ear mucosal (MEM) biopsies of children with OME and recurrent otitis media, using fluorescent in situ hybridization (FISH) and confocal scanning electron microscopy (CLSM), whereas biofilms were not observed in control MEM biopsies obtained from patients undergoing cochlear implantation [11]. Later, biofilm formation was also confirmed in middle ear mucosa of patients with COME [16].

Biofilms, however, do not only attach to tissue surfaces, but also to mucus [17, 18].

Therefore, this study aimed to investigate the presence of otopathogenic bacteria, i.e. *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus and Streptococcus pneumonia* in the middle ear effusions (MEE) and adenoids of a strictly defined group of children with COME, and to specifically investigate whether these bacteria, especially *H. influenzae*, are organized in a biofilm in the middle ear fluid.

Materials and methods

Patients and sampling

Children between 12 months and 6 years of age, diagnosed with COME, and undergoing adenoidectomy and ventilation tube placement were included. COME was defined as persistent (3 months or longer) middle ear effusion without signs or symptoms of acute ear infection (fever, pain, ear discharge).

Exclusion criteria were usage of local or systemic antibiotics within 30 days before the sample collection, known immune deficiencies, craniofacial malformation, previous adenoidectomy, and participation in other clinical trials within the last 3 months.

Middle ear effusions (MEE) were collected by using a middle ear fluid aspirator with collector (Juhn Tym-Tab, Xomed Medtronic, Jacksonville, Florida). Before paracenthesis, the outer ear canal was cleaned and sterilized with 70 % ethanol for 90 s, which has previously been demonstrated to be an effective method [3].

Adenoid tissue was collected by conventional curettage adenoidectomy.

Sample processing

Culture and identification by means of MALDI-TOF MS

Culture

Each middle ear effusion sample was used to inoculate two CHOC plates (Becton–Dickinson, Erembodegem, Belgium), 10 µl per plate. These plates were incubated aerobically with 5 % CO_2 and anaerobically (10 % H_2, 5 % CO_2 in N_2) for 5 days.

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. 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 min, whereafter it was vortexed again and two aliquots of 25 µl were inoculated onto two CHOC-plates. The broth suspension was then further incubated anaerobically. 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 two aliquots of 25 µl were inoculated onto two CHOC-plates and incubated as described above.

When more than one colony type appeared from a single effusion, each colony type was subcultured on CHOC plates and identified.

Identification by means of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOFMS) (Microflex Biotyper™, Bruker Daltonics, Bremen, Germany) was used to identify bacteria from MEE and adenoid samples, using the 'direct transfer' method, according to manufacturer's instructions.

Genotyping

Randomly amplified polymorphic DNA analysis (RAPD), in combination with melting curve analysis of the amplified DNA fragments (McRAPD) was carried out as described previously [19] to genotype strains when the same of one of the four otopathogenic species was found in both the adenoid and the MEE of at least one ear or in the MEE of both ears (even when the adenoid was culture negative).

Fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) of middle ear fluid

Because *Haemophilus influenzae* was the most frequently cultured bacterium, FISH was focused on this species.

For FISH, a universal bacterial probe targeting a conserved region of the 16S rRNA gene (EUB338 Probe, 3′CTGCCTCCCGTAGGA5′-Alexa488) [20] and a *H. influenzae* species specific probe (HAIN16S1251 Probe, 3′TCGCAGCTTCGCTTCCCT5′-Alexa647) [21] were used (Eurogentec, Seraing, Belgium).

Approximately 50 µl of the MEE samples were transferred to a 0.6 ml Eppendorf tube. These samples were washed during 5 min (with regular manual invertion of the tubes) with a total volume of 500 µl of 112 mM NaCl, 20 mM Tris–HCl (pH 8) and 0.01 % SDS (all Sigma-Aldrich, Diegem, Belgium). Wash buffer was removed by centrifugation at 11,500×*g* for 2 min. Samples containing blood were additionally treated for 10 min with 400 µl acetic acid (6 %) and centrifuged at 11,500×*g* for 2 min. This was repeated until all the blood present in the samples was removed. Subsequently, the sample was washed with 500 µl of washing buffer to remove the residual acetic acid. Prior to incubation with the probes, the samples were dehydrated by adding 300 µl of respectively 70, 85 and 100 % ethanol during 1 min and removal by 1 min of centrifugation at 11,500×*g*. Simultaneous hybridisation with 100 µl each of both probes was carried out at a final concentration of 200 nM of each probe in 20 mM Tris–HCl (pH 8), 30 % formamide (Sigma-Aldrich) and 0.01 % SDS, in a total volume of 200 µl, and incubated for 5 min at 80 °C in the dark, to denature the DNA, followed by incubation during 16 h at 50 °C in the dark, to enable the hybridization of the probes. After hybridization the samples were centrifuged at 11,500×*g* for 2 min and with 500 µl of washing buffer supplemented with 3 % Triton X-100 (Sigma-Aldrich) for 4 min at room temperature. Washing buffer with 3 % Triton X-100 was removed by centrifugation, and the samples were subsequently washed a second time with 500 µl of washing buffer with 3 % Triton X-100 at 73 °C for 2 min. Washing buffer was removed by centrifugation at 11,500×*g* for 2 min, and one cycle of washing at room temperature. Finally, the sample was dehydrated (as described above), stained with 200 µl of 1 µg/ml DAPI (Sigma-Aldrich) for 5 min in the dark, washed once and dehydrated again (see above). A small part of the middle ear fluid sample was transferred to a microscope slide and covered with a cover slide. The different samples were analyzed on a Leica SPE confocal microscope (Leica Microsystems, Wetzlar, Germany).

Results

Patients

In total, 21 patients were included in this study, 10 boys and 11 girls. The mean age was 3.3 years, with a minimum age of 1.1 year and maximum age of 6.6 years.

Culture

Culture results (Table 1) are presented for *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, the most common otopathogens in otitis media [20].

Table 1 Results of culture and genotyping isolates of the same bacterial species from different sites per patient

	Adenoid	Left ear	Right ear
1	SP a	**HI a**, HI b	**HI a**, MC, SP b
2	**HI c**, HI d, HI e, SP c	**HI c**, Hi f	HI NT
3	HI g, MC a	NG	SP d
4	**HI h**, **HI i**, SP d	NG	**HI h**, **HI i**
5	MC b, SP e	NG	NG
6	HI j, MC c, SP f	O	O
7	HI k, MC d, SP g, SP h, SP i, SP j	SP k, SP l	NG
8	**HI l**, MC e, SP m	**HI l**	NG
9	**HI m**, HI n, SP n	N	**HI m**, HI o
10	SP o	HI p	N
11	**HI q**, HI r, MC f, **SP p**, **SP q**	N	**HI q**, HI s, MC g, **SP p**, **SP q**
12	**HI t**, HI u, HI v, HI w, HI x, Hi y	Hi y	**HI t**, Hi z
13	HI a2, MC h, SA a, SP r	NG	N
14	SP s	NG	O
15	SP t	O	NG
16	HI b2, MC g, O	NG	N
17	Sp u	NG	N
18	SP v	SP v	N
19	MC i, SP w	O	NG
20	**HI c2**, **SP x**	**HI c2**, **SP x**	**SP x**
21	MC j, SA b	SA c, O	N

Focusing on *H. influenzae* (HI), *S. pneumoniae* (SP), *M. catarrhalis* (MC) and *S. aureus* (SA). When different genotypes were found, different alphabet letters were given. *NG* no growth, *N* no sample (unilateral OME). Bold indicates same genotypes in both MEEs or MEE and adenoid. *NT* genotype not tested, *O* other bacteria cultured

Middle ear effusions

In total, 34 MEE samples were collected from 21 patients (13 with bilateral OME, eight with unilateral OME), of which 22 samples (64.7 %) were culture positive for bacteria and 16 MEE samples (47 %) were culture positive for *H. influenzae*, *M. catarrhalis*, *S. aureus* and/or *S. pneumoniae*. *H. influenzae* was found most frequently (35.3 % of the MEE samples).

Seventeen out of the 21 (81.0 %) patients had at least one MEE with a culture positive result. In 13/21 patients (61.9 %), the MEE of at least one ear was culture positive for one or more of the four abovementioned species.

Adenoids

All 21 adenoid samples were culture positive for bacteria and all were culture positive for *H. influenzae*, *M. catarrhalis*, *S. aureus* or *S. pneumoniae*.

The most frequent species was *S. pneumoniae*, which was present in 16 out of 21 adenoid samples (76.2 %). *H. influenzae* was present in 12 out of 21 samples (57.1 %).

Relation between the presence of bacterial species in MEE and adenoid samples

When a positive MEE culture was obtained for one or more of the studied pathogens, at least one of these pathogens could also be cultured from the adenoid in 11 out of the 13 patients (84.6 %), and one of the considered pathogens could also be cultured from both the adenoid and the other MEE for two out of 13 patients. In one patient *H. influenzae* was cultured from both middle ears, but not from the adenoid.

Genotyping

Genotyping was only performed when the same bacterial species was found in both the adenoid and the MEE of at least one ear or in the MEE of both ears (even when the adenoid was culture negative). There were ten such cases for *H. influenzae*, four for *S. pneumoniae* and one each for *M. catarrhalis* and *S. aureus*. In all of the ten cases whereby *H. influenzae* was present in more than one location, at least one of the isolates in each location had the

same genotype of one of the isolates in the other location. Also in three of the four *S. pneumoniae* cases the isolates were from the same genotype. In the single cases with *M. catarrhalis* and *S. aureus*, different genotypes were found. In summary, for 13 of the 16 (81.25 %) cases whereby *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and/or *S. aureus* were isolated from more than one location, we could show the presence of identical strains in these locations. In the one case (patient 20) whereby *S. pneumoniae* was isolated from all three locations, it was also the same genotype in all three locations.

Fluorescence in situ hybridisation (FISH) and confocal laser scanning microscopy (CLSM)

FISH and CLSM were performed to detect biofilm formation. A universal bacterial probe and a species specific probe for *H. influenzae* were used, as *H. influenzae* was the most commonly cultured bacterial species in our MEE samples, and has also previously been reported to be implicated in biofilm formation in chronic middle ear inflammation, both in vitro [22] and in vivo [15].

Fourteen MEE samples of ten patients were selected for FISH and CLSM: 11 MEE samples, that were culture positive for at least *H. influenzae*, and three MEE samples (8R, 15R, 15L), that were culture negative for all four otopathogens, were used as negative controls.

In all of the 14 MEE samples, blood was visually present before the blood removal procedure was run. In four of these (8L, 8R, 11R and 12R), blood was still visible after the removal procedure and consequently FISH analysis was only possible for eight pathogen culture positive and two pathogen culture negative samples. Results from FISH and CLSM of these ten samples are summarized in Table 2.

In five of the eight pathogen culture positive MEE samples, *H. influenzae* specific aggregates, indicative for biofilms were present (Fig. 1a–c). In an additional culture positive MEE sample (20 L), non-specific bacterial clusters were observed (Fig. 1d). In none of the *H. influenzae* culture negative samples, *H. influenzae* specific aggregates were detected upon CLSM. However, non-specific bacterial clusters were detected in one *H. influenzae* culture negative sample (patient 15R) (Fig. 1d).

Table 2 FISH and CLSM results, *R*: right middle ear, *L* left middle ear

Sample	Presence of *H. influenzae* specific aggregates	Presence of bacterial aggregates other than *H. influenzae*
1R	Yes	No
2R	Yes	No
2L	No	No
4R	No	No
9R	Yes	No
10L	Yes	No
12L	Yes	No
20L	No	Yes
15R[a]	No	Yes
15L[a]	No	No

[a] Culture negative for the 4 otopathogens

Discussion

Otitis media with effusion is a highly prevalent pediatric disease, associated with significant morbidity, socio-economic impact and impairment of quality of life. This disease is characterized by frequent spontaneous resolution but also by a high recurrence rate. Medical treatment has no long term beneficial effect. In protracted cases or cases with important hearing loss, placement of ventilation tubes is advised [23].

A possible explanation for the poor response to antibiotics and the possible progression of OME to a chronic disease is the involvement of biofilms [8]. Planktonic shedding, part of the life cycle of biofilms, whereby bacteria detach from the biofilm and return to a planktonic phenotype, can cause bouts of acute otitis with signs and symptoms of acute disease sometimes warranting antibiotic treatment.

In this study, we first investigated the relation between otopathogenic bacteria in MEE and the adenoid in children with COME.

Of all MEE samples, 65 % were culture positive for bacteria and 47 % culture positive for *H. influenzae*, *M. catarrhalis*, *S. aureus* and/or *S. pneumoniae*. The most frequently found bacterial species in the MEE samples was *H. influenzae*, which was found in 35 % of MEE samples. In 13 of the 16 (81.25 %) cases whereby *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and/or *S. aureus* were isolated from more than one location, we could show the presence of identical strains in these locations. Although it was not the primary goal of this study to show that the adenoid may act as a reservoir for bacteria involved in COME, our data seem to support this hypothesis, but the limited number of patients does not allow us to draw firm conclusions.

As in other studies [3, 20, 24, 25], *H. influenzae* was the most frequently cultured otopathogen, although we found a higher number of positive cultures for otopathogens in our middle ear samples, compared to other studies, where bacteria were culturable in less than 60 % of OME samples [3, 20, 24, 25]. This could be explained by the younger age of our patients (all between 1 and 6 years old) compared to other studies that also included older children or even adults, where it was found that children appeared to have a greater number of culture positive MEEs compared to

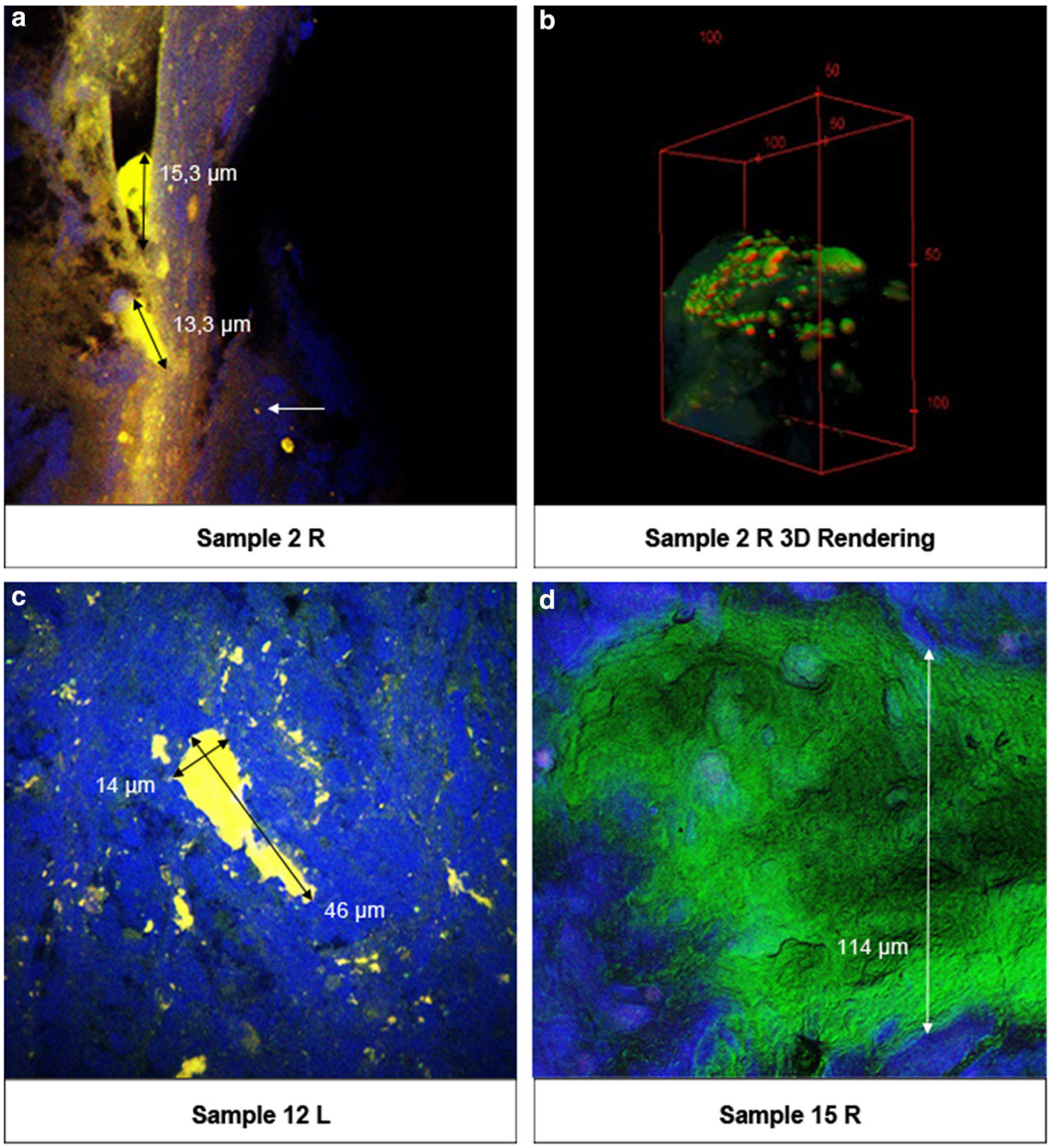

Fig. 1 CLSM images of biofilm structures visualized by FISH. **a** Two neighboring *H. influenzae* biofilm structures of 15.3 and 13.3 µm with planktonic bacteria (*arrow*). These are part of a larger polymicrobial biofilm structure visualized in 3D (**b**). **c** A large biofilm structure of *H. influenzae* bacteria of 46 × 14 µm with planktonic bacteria around the biofilm structure (*arrows*). **d** No *H. influenzae* was detected in this culture negative sample (15R), which acted as a negative control. The *green* staining suggests that biofilm structures are present in this sample, which do not contain *H. influenzae*. Two probes were used for the visualisation of biofilms in MEE samples: EUB388-Alexa555 probe is a universal probe which stains all bacteria green. The *H. influenzae* specific probe stains *H. influenzae* bacteria red. The combination of the EUB388-Alexa555 probe and the *H. influenzae* specific probe results in a *yellow colour*, which specifically indicates the presence of *H. influenzae*. DAPI (4′,6-diamidino-2-phenylindole) pigment stains eukaryote nucleoli blue

adults with COME [3, 24, 25]. Also, our strict exclusion of patients that used topical (nose and/or ear) or systemic antibiotics within 30 days before the sample collection could explain our higher rate of culture positive middle ear samples compared to studies that did not exclude antibiotic usage prior to MEE sampling [24, 25]. In addition, the culture techniques can influence the culture results. In our study all effusion samples were cultured on chocolatised blood agars, aerobically and anaerobically. Daniel et al. [3] for example cultured the MEEs on six different media and also performed aerobic and anaerobic culturing, but anaerobic culturing was only performed with sheep blood agar, which is not suited to culture *H. influenzae*, which might explain the very low number of *H. influenzae* isolates

in their samples. Finally, in accordance with the assumption that bacteria in COME are organized in a biofilm, it is possible that also the sampling with an aspirator-collector and the further sample processing could have influenced the results by disrupting the biofilm, therefore yielding higher culture positive results.

The value of culture techniques in assessing the pathophysiological role of bacteria in otitis media (with effusion) has much been debated. On the one hand, culture of bacteria is supposed to underestimate the presence of bacteria in MEE of OME. Positive culture rates vary from 20 to 60 % throughout the studies, whereas PCR (polymerase chain reaction) techniques typically detect bacterial DNA in more than 80 % of MEE from patients with COME [6]. On the other hand, the presence of bacterial DNA does not necessarily indicate the presence of living bacteria, as bacterial DNA can persist for a long period even if the bacteria are no longer viable, which of course also questions the reliability of PCR techniques in analyzing the pathophysiological role of bacteria. We did not perform PCR, as the sample volumes were too small to perform both qPCR and FISH. A selection of effusions were analyzed to investigate whether these bacteria organize into biofilms in the middle ear fluid, we performed FISH with both a universal bacterial probe and a species specific probe for *H. influenzae*. In 50 % of the analysed samples, *H. influenzae* specific aggregates, indicative for biofilm formation, were detected by CLSM. In an additional 20 % of the samples, bacterial aggregates by other bacteria than *H. influenzae*, indicative for biofilm formation by other undefined bacteria, was found, resulting in 70 % of the analysed MEE samples showing biofilm formation. Daniel et al. [3] found very similar results and detected biofilms by CLSM in the middle ear fluid of 67.9 % of children with OME, while, on the other hand, they could not document biofilm in any of the adult samples.

In none of our *H. influenzae* culture negative samples, *H. influenzae* specific biofilm structures were detected, whereas in 62.5 % of *H. influenzae* culture positive samples, *H. influenzae* specific biofilms structures were seen, which indicates that extensive culture techniques do give an indication of bacterial species that might be involved in biofilm formation. This contrasts with previous statements that bacteria in biofilms are not culturable [26]. As mentioned above, however, we have to consider the possibility that the large number of positive cultures result from biofilm destruction due to sampling and laboratory processing techniques [8]. On the other hand, sampling and processing can also lead to an underestimation of biofilm formation, as a biofilm can be localised in only part of the MEE, or the biofilm may have been be disrupted.

In conclusion, we found a high number of bacterial aggregates, indicative of in vivo biofilms in MEE of children with chronic otitis media with effusion. This supports the biofilm paradigm in chronic otitis media with effusion, at least in young children, and confirms that biofilms can also be formed in the middle ear fluid and not only on the middle ear mucosa [11]. These findings improve insights in the pathogenesis of otitis media with effusion and may help to develop future treatment strategies.

It remains to be established that *H. influenzae* is indeed the most important bacterial species involved in biofilm formation in COME, as other (oto)pathogens might also play a major role. At the level of the adenoid, it should be assessed whether the supposed bacterial reservoir for the middle ear also organizes into biofilms, and if so, how exactly this influences the middle ear status. Finally, it needs to be confirmed whether biofilm formation in COME is indeed limited to the pediatric patient group, which could indicate that COME in adults represents a different disease entity.

Conclusions

The findings in this study indicate that the adenoid acts as a reservoir for bacteria in MEE. Secondly this study confirms that biofilms, specifically consisting of *H. influenzae*, are indeed 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.

Compliance with ethical standards

The authors have no conflict of interest. No external funding was provided for this study. The study was approved by the Ethical Committee of Ghent University Hospital (number of approval: B670201214394). Written informed consent was provided by the parents or legal guardians of the study participants.

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