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REVIEW ARTICLE

Otitis media pathogens – A life entrapped in biofilm communities

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ABSTRACT

Otitis media is a group of inflammatory diseases of the middle ear with great impact on children worldwide. The most common reported bacterial pathogens are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. Over the last years, the role of biofilms formed by otopathogens that contribute to otitis media recurrence and chronicity has been established. An improved understanding of the properties of biofilms formed by these bacteria, which factors influence them, and how these affect the host inflammatory response is important for the development of novel strategies for the treatment of otitis media. This review focuses on the biofilm nature that the most prevalent otopathogens adopt in otitis media infections. In addition, new treatment approaches targeting biofilms are highlighted.

Introduction

Otitis media (OM) is a group of inflammatory disorders that affects the middle ear (ME) (Figure 1), being one of the most common diseases in childhood and a leading cause for medical consultation, antibiotic prescription and surgery procedures in young children. Different types of OM exist and are distinguished based on the presence of fluid in the ME with or without inflammation, the manifestation of symptoms and the appearance or perforation of the tympanic membrane (Schilder et al. 2016; Bergenfelz and Hakansson 2017).

Acute otitis media (AOM) is characterized by the rapid accumulation of fluid in the ME, with signs and symptoms of an acute infection, such as otalgia, otorrhea, fever and vomiting (Massa et al. 2009; Schilder et al. 2016). Globally, approximately 709 million cases of AOM occur each year, 51% of which affect children under 5 years-old (Monasta et al. 2012). The occurrence of more than 3 episodes of AOM in 6 months or of 4 or more episodes in 12 months is defined as recurrent AOM (RAOM), that is very common, causing much suffering for children and their parents (Schilder et al. 2016). For instance, the RAOM rate in children aged 3 and 5 years is estimated at about 50% and 65%, respectively (Teele et al. 1989). Recurrence is very common in children attending day care centers, but also

other risk factors (described below) can be associated with this occurrence (Schilder et al. 2016). Acute mastoiditis, a relatively rare but more serious complication of AOM, can also occur when the infection in the ME spreads to the mastoid air cells and covers the periosteum (Qureishi et al. 2014).

Otitis media with effusion (OME) is characterized by the presence of a glue-like fluid in the ME behind an intact tympanic membrane with no signs and symptoms of an acute infection. In a systematic literature review analyzing data from 63 different articles, it was shown that 28% cases had a spontaneous resolution by 3 months and increased to 42% by 6 months (Rosenfeld and Kay 2003). The most common symptom of OME is hearing loss, caused by an impaired transduction of sound waves due to the presence of ME effusion (MEE). Persistence of OME for 3 months or more is considered a chronic condition named chronic otitis media with effusion (COME). A persistent hearing loss may negatively impact speech development, behavior and progress at school (Qureishi et al. 2014; Schilder et al. 2016). OME is asymptomatic, and therefore the accurate determination of its incidence and prevalence is difficult. Nonetheless, estimates show that around 90% of children experience at least one case of OME before the school age (American Academy of Family Physicians et al. 2004).
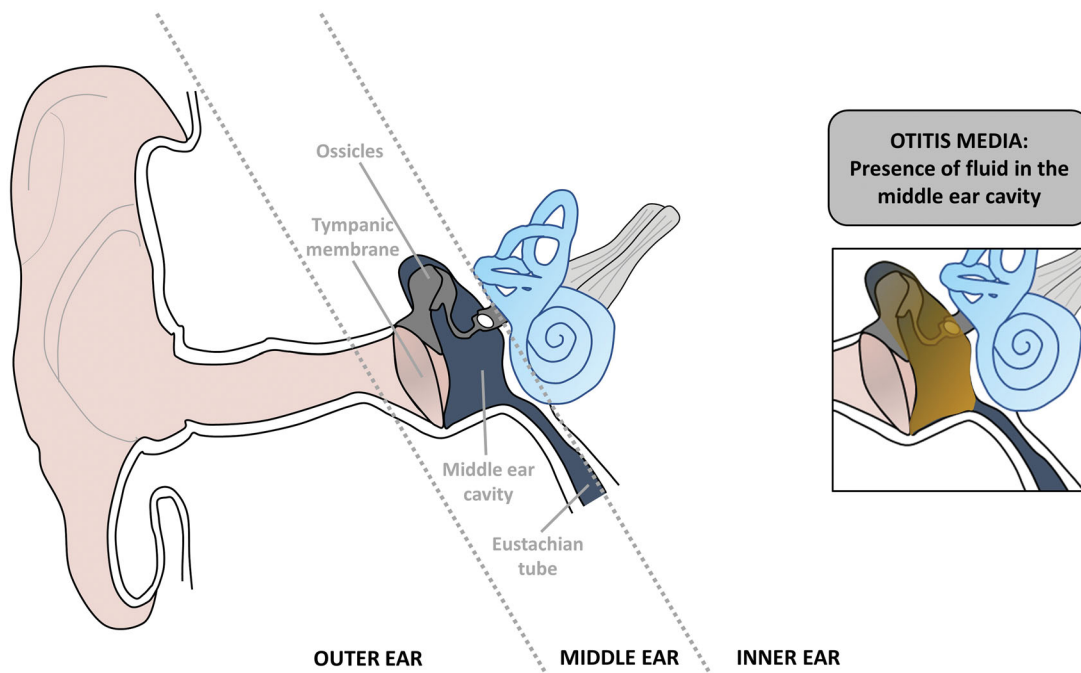
Figure 1. Anatomy of the human ear (outer, middle and inner ear) and representation of otitis media.

Chronic suppurative otitis media (CSOM) is based on the persistent presence of purulent MEE with, ordinarily, ear discharge through a tympanic membrane perforation (Qureishi et al. 2014; Schilder et al. 2016). Approximately 31 million cases of CSOM occur each year worldwide, with 22.6% affecting children under 5 years of age (Monasta et al. 2012).

Another OM complication is cholesteatoma, which is characterized by the presence of keratinizing squamous epithelium in the ME, manifesting as a chronic ear smelly ear discharge (Qureishi et al. 2014).

This review focuses on AOM and OME, their respective recurrence or chronicity and the microbiology associated due to their impact on children.

Microbiology of otitis media

Bacteria and viruses, individually or together, are associated with OM development. *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* are the most frequently identified otopathogens (Ngo et al. 2016). Implementation of pneumococcal conjugate vaccines (PCVs) into national immunization programs has resulted in important changes in the microbiology of OM. *H. influenzae* detection surpassed *S. pneumoniae*, becoming the most common causative agent worldwide. Furthermore, non-vaccine covered *S. pneumoniae* serotypes became the most commonly isolated serotypes from OM cases (Ngo et al. 2016, Dagan et al. 2016). These bacterial species colonize the nasopharynx since early infancy and are considered part of the commensal flora, causing no harm until alterations occur in the nasopharyngeal milieu (Marom et al. 2012). Viruses, such as adenovirus, influenza virus types A and B, respiratory syncytial virus, rhinovirus and parainfluenza virus, are also causative agents of OM (Chonmaitree et al. 2008).

Bacterial nasopharyngeal colonization transition to ME inflammation is highly correlated with concomitant upper viral respiratory tract infection (Heikkinen and Chonmaitree 2003). Viral infection of the nasopharynx alters the environment in this mucosa, modifying host immune function, inducing cytokine activity and inflammatory mediators, and increasing bacterial colonization and adherence to host cells through the upregulation of antigens that act as bacterial receptor sites (Bakaletz 2010). The properties of the mucus and its clearance by cells of the Eustachian tube (ET) and nasopharynx are also altered by the viral infection causing ET dysfunction and obstruction, originating a negative pressure in the ME that facilitates the influx of microorganisms to the ME cavity. Once in the ME, an inflammatory condition is established, inducing the disease (Marom et al. 2012). In a prospective longitudinal study of almost 300 children, OM incidence following an upper respiratory viral infection was of 61%, with an AOM incidence of 37% and an OME incidence of 24% (Chonmaitree et al. 2008).

Infants and young children are more susceptible to OM due to the shorter, wider and more horizontal anatomy of the ET (Figure 2). The ET is responsible for the equalization of pressure, protection of the ME against
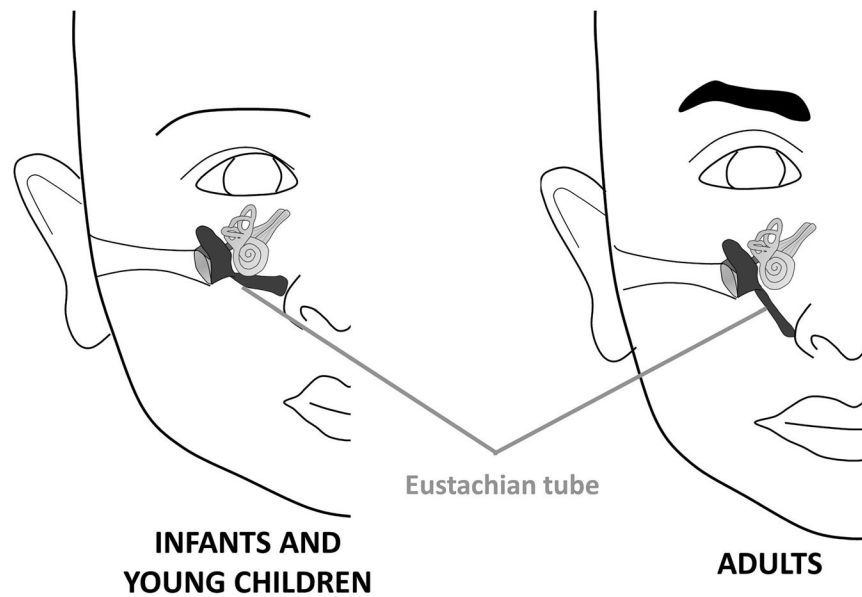
Figure 2. Differences in the anatomy of the Eustachian tube in infants and young children versus adults.

the influx of pathogens, and clearance of secretions. As the child grows, the ET matures, as well as the immune system, decreasing the risk of OM (Rovers et al. 2004; Qureishi et al. 2014; Schilder et al. 2016).

As a multifactorial disease, the risk of OM increases with many other aspects. These include host features, such as young age, male gender, genetics, family history of OM, craniofacial anomalies, allergies, immunodeficiency, and others. Also, environmental factors, including day care attendance, low socioeconomic status, tobacco smoke exposure, short duration of breastfeeding, use of pacifiers, among others, are associated with an increased incidence of OM (Rovers et al. 2004; Bakaletz 2010; Zhang et al. 2014; Kørvel-Hanquist et al. 2018).

The biofilm nature of otitis media

Over the last years the role of biofilms in OM has been demonstrated. Biofilms are communities of microorganisms embedded in a self-produced extracellular polymeric matrix (EPS) and attached to a surface (Figure 3) (Donlan 2002). Growth in biofilms is advantageous, protecting bacteria from environmental stresses, host immune defenses, and antimicrobial agents. Lower growth rates of bacteria within biofilms contribute also to their increased antimicrobial resistance (Donlan 2002; Stoodley et al. 2002). Metabolically inactive cells, called persister cells, play a major role in the recalcitrance of biofilms to antimicrobials (Lewis 2010). Biofilm formation is largely associated with an alteration in gene expression, with specific genes down- or up-regulated under control of a gene regulation system known

as quorum sensing (QS) (Donlan 2002; Stoodley et al. 2002).

The biofilm nature of OM hypothesis evolved from a set of observations (Table 1). For many years, ME effusions collected from patients that were culture-negative were considered sterile. However, a significant percentage of bacterial DNA is today detected by polymerase chain reaction (PCR) methods in culturally sterile ME fluids (Post 1995). In a chinchilla model of OM, viable antibiotic-treated bacteria were detected by PCR in contrast to purified DNA and DNA from intact but non-viable bacteria, which were not detected even following high copy inoculation (Post et al. 1996). Furthermore, the presence of bacterial mRNA by reverse-transcriptase PCR (RT-PCR) in culture negative otitis media effusions suggested the existence of metabolically active bacteria (Rayner et al. 1998). Formation of a *H. influenzae* biofilms on the ME mucosa of chinchilla after bacterial injection reinforced that biofilms may play a role in the etiology of OM (Post 2001). Microscopy detection of biofilms on ME mucosa biopsy samples also supports the hypothesis that these disorders may be biofilm related (Hall-Stoodley et al. 2006).

Biofilm formation by pathogenic bacteria in the nasopharynx followed by shedding of biofilm cells has also been pointed as an important contributor to OM, particularly AOM. Dispersed cells may enter into the ME cavity, taking advantage of the negative pressure in the ME due to ET dysfunction. Once in the ME, these bacteria can establish a new biofilm and induce disease. Since bacteria in biofilms can resist to the antimicrobial agents administered more effectively,

Table 1. Studies supporting the hypothesis of the biofilm nature of OM.

Sample	Origin	Species	Main results	Ref.
MEF	Children with COME	H. influenzae, S. pneumoniae, M. catarrhalis	28.9% of samples culture positive/PCR positive; 48% of samples culture negative/PCR negative.	(Post 1995)
MEF	Chinchilla model[a]	Set 1: live H. influenzae, pasteurized M. catarrhalis and purified S. pneumoniae DNA Set 2: live S. pneumoniae, pasteurized M. catarrhalis and purified H. influenzae DNA	PCR negative for detection of pasteurized bacteria or purified DNA after day 3; PCR positive/culture negative for detection of live bacteria until day 21.	(Post et al. 1996)
MEF	Children with COME	H. influenzae	11.8% of samples culture positive/RT-PCR positive; 31.2% of samples culture negative/RT-PCR positive.	(Rayner et al. 1998)
MEM	Chinchilla model[a]	H. influenzae	Bacterial biofilms with increasing density and thickness as time progresses observed on MEM of chinchillas with experimental OM; control chinchillas didn't show biofilms on MEM.	(Post 2001)
MEM	Children with COME or RAOM	H. influenzae, S. pneumoniae, M. catarrhalis	Biofilms in 92% of samples that varied in morphology	(Hall-Stoodley et al. 2006)

MEF: middle ear fluid; MEM: middle ear mucosa (biopsy samples); COME: chronic otitis media; RAOM: recurrent acute otitis media.
[a]Experimentally infected via transbullar injection.

recurrent episodes of OM (RAOM) are probable to occur (Coticchia et al. 2013).

Importantly, it is hypothesized that there is a continuum from early AOM episodes to more complex cases of COME that are usually linked with polymicrobial biofilms. Children had their first AOM case due to some genetic or environmental factor, with invasive strains of *S. pneumoniae* being frequently involved. This initial episode causes some mucosal damage that may predispose children to subsequent infections induced by pneumococcal serotypes with lower disease-inducing potential and other bacterial species such as *H. influenzae* and *M. catarrhalis*, with increased biofilm formation and polymicrobial interaction ability (Dagan et al. 2016).

In vitro and in vivo biofilm studies

Haemophilus influenzae

Several studies characterizing the biofilm phenotype of *H. influenzae* have been performed, using different microscopy techniques *in vitro* and *in vivo* (Table 2). Also, the role of diverse compounds from both bacterial and host origin that seem to impair in *H. influenzae* biofilm phenotype and their impact in OM development and progression will be discussed.

H. influenzae strains ability to form robust biofilms with complex structures has been demonstrated in the ME of chinchilla. For instance, an increased bacterial density and development of a matrix-encased biofilm, consistent with the progression of time, was observed by scanning electron microscopy (SEM) in ME mucosal

biopsy samples (Post 2001), or SEM and confocal laser scanning microscopy (CLSM) (Ehrlich et al. 2002). In the latter work, animals were killed at different time points with ME mucosal biopsy samples showing the evolution of biofilms from microcolony formation, characteristic of an early biofilm, to mature biofilms containing viable cells with an increased thickness and presence of water channels (Ehrlich et al. 2002).

ME mucosal biopsy samples of children with COME presented biofilms as demonstrated using generic stains and species-specific probes. Confocal images of the *H. influenzae* PCR positive samples showed matrix-enclosed bacterial clusters, with a range of biofilm morphologies as observed by fluorescence *in situ* hybridization (FISH), (Hall-Stoodley et al. 2006). Biofilms formed by ME pathogens, including *H. influenzae,* were observed by SEM and CLSM coupled to FISH in the adenoids of children with RAOM, suggesting that biofilms in the nasopharynx act as reservoir for AOM recurrence (Hoa, Tomovic, et al. 2009). The presence of *H. influenzae* biofilms on the adenoids (Nistico et al. 2011) and in ME effusion samples (Van Hoecke et al. 2016) from patients with COME has also been reported. These data are in accordance with studies that report a stronger association of *H. influenzae* with RAOM and COME events. Although less-severe symptoms are detected, these cases are more difficult to treat and resolve properly (Barkai et al. 2009, Stol et al. 2013).

Besides addressing the presence of biofilms in OM samples, a few studies have also focused on the biofilm forming capacity of *H. influenzae* (Supplemental Table S1). Nontypeable *H. influenzae* (NTHi) clinical isolates

Table 2. Microscopic detection of biofilms associated with otitis media.

Sample	Origin	Species	Main results	Ref.
MEM	Chinchilla model[a]	H. influenzae	Bacterial biofilms with increasing density and thickness as time progresses observed on MEM of chinchillas with experimental OM; control chinchillas didn't show biofilms on MEM.	(Post 2001)
MEM	Chinchilla model[a]	H. influenzae	Biofilms in all samples collected from day 1 to 21. Bacteria within biofilms were viable.	(Ehrlich et al. 2002)
MEM	Children with COME or RAOM	H. influenzae, S. pneumoniae, M. catarrhalis	Biofilms on 92% of samples that varied in morphology.	(Hall-Stoodley et al. 2006)
MEM	Chinchilla model[a]	S. pneumoniae	Dense 3D structures containing viable cells, and viable and non-viable host cells attached to a fibrous DNA matrix.	(Reid et al. 2009)
Adenoids	Children with RAOM	H. influenzae, S. pneumoniae, M. catarrhalis, S. aureus	Biofilm coverage higher than 86% in all samples. Positive FISH staining for at least one of the pathogens	(Hoa, Tomovic, et al. 2009)
Adenoids and MEM	Chinchilla model[a]	S. pneumoniae	Biofilms in NP (83%) and MEF (67%) samples in chinchillas with confirmed middle ear infection. All animals with MEM biofilms had biofilms in the NP.	(Hoa, Syamal, et al. 2009)
Adenoids	Children with COME or OSA	Several	Presence of several pathogens, with higher frequency of H. influenzae in COME cases than OSA. Aggregates of viable bacteria encased in an extracellular matrix consistent with biofilm structures.	(Nistico et al. 2011)
MEF and adenoids	Children with COME	H. influenzae	H. influenzae specific aggregates, indicative of biofilms, present in 5 out of 8 culture positive samples, but in none of the culture negative samples.	(Van Hoecke et al. 2016)

MEF: middle ear fluid; MEM: middle ear mucosa (biopsy samples); NP: nasopharynx; COME: chronic otitis media; RAOM: recurrent acute otitis media; OSA: obstructive sleep apnea.
[a]Experimentally infected via transbullar injection.

had a remarkable variability in their capacity to form *in vitro* biofilms, with P2, P5 and P6 outer membrane proteins expressed during biofilm growth (Murphy and Kirkham 2002). In another study, more than 80% of NTHi isolated from AOM cases were shown to be good biofilm forming strains. Furthermore, the biofilm forming capacity of identical strains isolated both from ME fluid and nasopharynx was well correlated (Moriyama et al. 2009). Another interesting study showed that NTHi isolates collected from patients with invasive diseases and OM had a significant ability to form biofilms compared to isolates from other respiratory disease cases and healthy subjects, demonstrating that biofilms do play an important role on OM (Puig et al. 2014). In contrast, no association between biofilm formation by NTHi strains and AOM treatment failure or recurrence has also been reported (Mizrahi et al. 2014).

Several studies address the influence of specific moieties of lipooligosaccharides (LOS) on *H. influenzae* biofilms. *H. influenzae* is not able to synthetize sialic (N-acetyl-neuraminic) acid but can acquire it from environmental sources and incorporate this important compound into its LOS structures. Sialylated LOS on

NTHi surface promotes biofilm formation *in vitro* (Swords et al. 2004). The NTHi 2019 strain produces a biofilm containing α2,6-linked sialic acid with levels of sialylated LOS increasing during biofilm growth (Greiner et al. 2004), and mutations in *siaA, siaB,* and *wecA* resulted in diminished biofilm formation while mutations in *pgm, lic3A* and *lsgB* resulted in equivalent or greater biofilms compared to NTHi 2019. A similar study performed in a chinchilla OM model showed that the wild type strain formed a large, well-organized, and viable biofilm, while mutants were either unable to form biofilms or formed biofilms of markedly reduced mass, organization, and viability (Jurcisek et al. 2005). Furthermore, some of the mutants survived in the chinchilla, inducing culture-positive ME effusions, while others were extremely sensitive to the bactericidal activity of chinchilla serum, indicating that LOS sialylation is indispensable for survivability of NTHi *in vivo* (Jurcisek et al. 2005).

Besides sialic acid, the impact of LOS containing phosphorylcholine (PCho) on *H. influenzae* biofilms has been investigated. CLSM analysis of NTHi biofilms grown on epithelial cell surfaces revealed that LOS

sialylated variants were distributed throughout the biofilm, while LOS variants expressing PCho were found within the biofilm (West-Barnette et al. 2006). Furthermore, these authors showed that LOS purified from NTHi biofilms or PCho positive strains were significantly less potent inducers of inflammatory responses than LOS purified from planktonic cultures or PCho negative strains, possibly justifying the quiescent periods of low-level inflammation that are typical of chronic NTHi infections. A study comparing the virulence of an OM isolate with that of a mutant lacking PCho showed that the mutant elicited an increased host inflammatory response early after challenge and induced a slower progression of disease in the chinchilla ME infection model (Hong, Mason, et al. 2007). In this study, differences in the presence and apparent density of biofilms formed in the ME of animals were noted, with PCho variants promoting biofilm formation. Similarly to the study by West-Barnette et al. (2006), NTHi biofilms formed within the chinchilla ME contained PCho positive variants within the biofilm structure. In a later study, the same research group concluded that PCho expression correlates with the maturation of NTHi biofilms, and may promote both *in vitro* and *in vivo* biofilm formation (Hong, Pang, et al. 2007).

Identification of proteins involved in biofilm formation and their distribution within biofilms is of utmost importance. For instance, peroxiredoxin-glutaredoxin protein was found to be present in greater abundance in *H. influenzae* biofilms compared to planktonic bacteria being directly involved in biofilm formation (Murphy et al. 2005). LOS were found to accumulate in the extracellular matrix at the base of the biofilm, with P6 outer membrane protein (OMP), a highly conserved protein in *H. influenzae* able to stimulate macrophage and epithelial cells, localized in the membrane of what appear to be viable bacteria within the biofilm (Webster et al. 2006). Hap and HWM1/HMW2 adhesive proteins were found associated with bacteria within the biofilm and in the EPS, while Immunoglobin A1 protease was more concentrated in the top region of the biofilm possibly having a role in protecting bacteria from antibody attacks (Webster et al. 2006).

The chinchilla ME infection model was used to demonstrate that NTHi biofilms contain type IV pili and a significant amount of double-stranded DNA that may be conferring structural stability to biofilms (Jurcisek and Bakaletz 2007). Later, the same research group reported that PilA protein, and by inference type IV pili, played an important role in NTHi biofilm formation and adherence to epithelial cells *in vitro*, and that *pilA* genes were transcribed during colonization and infection with

its expression being required for the formation of stable biofilms *in vivo* (Jurcisek et al. 2007). Another study also showed that the EPS matrix of NTHi biofilms is composed of proteins, nucleic acids and β-glucan, with eDNA being an important component of the EPS and essential in biofilm maintenance (Domenech et al. 2016). NTHi *in vitro* biofilms visualization combined with mathematical tools demonstrated that eDNA and type IV pili expression are both important for the development of fractal structures in these biofilms, and may help bacterial survival in hostile environments, such as the ME (Das et al. 2017).

The potential roles of neutrophil extracellular traps (NETs) by which neutrophils entrap bacteria by extrusion of their genomic DNA, aiming to kill them, has been observed for *H. influenzae* (Hong et al. 2009). Biofilms recovered from the ME of experimentally NTHi infected chinchillas showed viable host and bacterial cells within a fibrous DNA matrix and presence of elastase and histones within the biofilm structure, which is consistent with the existence of NETs. The contribution of LOS to *H. influenzae* survival in NETs was assessed by a bactericidal assay showing that NTHi with LOS mutations, in regions previously shown to promote biofilm formation, were more readily killed by NETs, and thus indicating that biofilm phenotype confers resistance to NETs (Hong et al. 2009).

The QS systems of *H. influenzae* have also been subject of research. The role of the *luxS*, the genetic determinant of the production of the auto-inducer-2 (AI-2) quorum signal, has been investigated, and the *luxS* mutant was found to produce an identical biofilm to that of the wild type strain but which tended to be more invasive to human cells, and also apparently more virulent in chinchilla (Daines et al. 2005). These authors concluded that the inactivation of the *luxS* gene results in dysregulation of pathways that are important in the pathogenesis of OM. In further studies, a significant reduction in thickness and overall density was observed in biofilms formed by the *luxS* mutant compared with the parental strain *in vitro*, and also in the chinchilla model (Armbruster et al. 2009), suggesting that *luxS* promotes biofilm maturation. Analysis of LOS composition by immunosorbent and immunoblotting assays revealed that *luxS* mutation resulted in a decreased content of PCho (Armbruster et al. 2009). RbsB protein was shown to mediate quorum signal uptake by binding to AI-2. Similarly to *luxS* mutants, *rbsB* mutants formed biofilms with reduced thickness and biomass, reduced PCho, and impaired bacterial persistence in the chinchilla ME, suggesting that AI-2 quorum signaling is used by NTHi to promote biofilm formation and establishment of chronic

infections (Armbruster et al. 2011). Recently, a glycosyl-transferase which expression is increased by AI-2 production by NTHi, being involved in the synthesis of biofilm matrix, was identified (Pang et al. 2018).

In addition to the QS mechanism regulated by AI-2 produced by *luxS,* the two-component signaling system QseB/C was also shown to be involved in NTHi biofilm formation. Deletion of the *qseC* gene resulted in a significant decrease of biofilm formation under different flow conditions, that were not correlated with AI-2 levels (Ünal et al. 2012).

The biofilm etiology in OM may explain the ineffectiveness of antibiotics in the treatment of this condition, due to the inherent inability of antibiotics to eradicate the biofilm bacteria. A decreased susceptibility of biofilm cells to antibiotics such as amoxicillin was shown using *H. influenzae* ME isolates (Slinger et al. 2006; Moriyama et al. 2009; Takei et al. 2013), with antibiotic combinations containing rifampin and ciprofloxacin being the most effective against *H. influenzae* biofilms (Slinger et al. 2006). Thus, it is important to continue to investigate efficient ways to eradicate this pathogen.

Streptococcus pneumoniae

Biofilm formation by *S. pneumoniae* has also been associated with OM. Herein, we will focus on studies performed over the last two decades that prove that *S. pneumoniae* forms well-organized biofilms both in the nasopharynx and in the ME, and further discuss the different factors that appear to influence pneumococcal biofilm formation and dispersal in these environments.

Microscopy techniques have shown pneumococcal biofilms both in the ME and nasopharyngeal samples (Table 2). *S. pneumoniae* biofilm structures were identified in the ME mucosa specimens of children diagnosed with COME. Analysis of *S. pneumoniae* PCR positive samples by CLSM revealed clusters of cocci apparently adhered to the ME mucosa and surrounded by an amorphous matrix. The presence of biofilms was also confirmed by FISH using a *S. pneumoniae* specific probe (Hall-Stoodley et al. 2006). The formation of *S. pneumoniae* biofilms in the ME of experimentally infected chinchilla was also reported. By different microscopic techniques, it was possible to observe viable pneumococcus, as wells as viable and nonviable host cells, entrapped in a fibrous matrix composed of DNA, indicating the existence of NETs (Reid et al. 2009).

Different microscopy techniques have been used to localize pneumococcal biofilms in samples collected from the adenoids of children with RAOM (Hoa, Tomovic, et al. 2009), and also in nasopharyngeal and

ME mucosal samples collected from infected chinchilla model after introducing a viral infection only then colonizing with the bacterial strain (Hoa, Syamal, et al. 2009).

Biofilm formation by *S. pneumoniae* has been a focus of study (Supplemental Table S2). The biofilm development process of distinct serotypes was characterized using a continuous-flow biofilm reactor system, and resulted in a three-stage biofilm formation that included initial attachment, cluster formation, and finally biofilm maturation (Allegrucci et al. 2006). This study showed that the architecture of the mature biofilms differed significantly among the serotypes tested and was correlated with proteomic changes, with a significant number of proteins detectable only on mature biofilms. Moreover, proteins involved in virulence, adhesion, and resistance were more abundant under biofilm growth conditions than planktonic conditions. More recently, protein profiles in planktonic and biofilm phenotypes were compared resulting in 80% of proteins differentially expressed during biofilm formation (Allan et al. 2014). These authors further observed that, in contrast to previously reported (Allegrucci et al. 2006), enzymes involved in the glycolytic pathway, in translation, transcription, and virulence were downregulated, while proteins associated with pyruvate, carbohydrate, and arginine metabolism pathways were more expressed during biofilm formation (Allan et al. 2014).

Studying gene products in biofilm formation demonstrated that choline-binding proteins are important for the biofilm development, with gene mutations resulting in decreased biofilm formation capacity (Moscoso et al. 2006). This study also showed that encapsulated strains have an impaired ability to form biofilms compared to non-typeable strains. Colony morphology variants with differences in colony size, capsule production and attachment occur in *S. pneumoniae* biofilm formation (Allegrucci and Sauer 2007). An analysis of biofilm-phase variants differing in colony morphology revealed that most had mutations in the *cps3D* gene of the capsular operon (McEllistrem et al. 2007). Emergence of *S. pneumoniae* biofilm cell variants differing in capsule production, attachment, and biofilm formation was reported to occur due to an increased mutation rate affected by environmental conditions (Allegrucci and Sauer 2008), suggesting that biofilm development tends to select for unencapsulated (non-typeable) phenotypic variants. Biofilms formed by encapsulated pneumococcal nasopharyngeal isolates, under conditions that simulate those in the ME during OME, presented a matrix composed of eDNA, and good biofilm formers further presented an enhanced structural complexity and increased antibiotic resistance

(Hall-Stoodley et al. 2008). In addition, the *cpsA* gene of the capsular operon was downregulated in all *S. pneumoniae* biofilms compared to planktonic living. Biofilm-forming capacity has been correlated with a reduction in colony size and in the relative amount of capsular polysaccharide present on the cell surface of *S. pneumoniae* biofilm colony variants (Domenech et al. 2009; Qin et al. 2013). Nonetheless, this was not a straightforward correlation since mutants with a low production of capsular polysaccharide and impaired biofilm formation were also found (Domenech et al. 2009). According to the authors, the heterogenous population was involved in different stages of the biofilm formation process, with the unencapsulated and good biofilm forming strains being essentially involved in the initial adhesion.

Today, there is sufficient evidence showing that eDNA, proteins and polysaccharides are, like in so many other bacterial species, also the main components of the *S. pneumoniae* biofilm matrix, and furthermore that strains with absent capsular polysaccharide form thicker biofilms (Domenech, García, et al. 2013). The reason why some specific serotypes are more prevalent in OM remains questionable. Capsular polysaccharide is considered the major virulence determinant of *S. pneumoniae*, existing more than 90 known serotypes (Gilley and Orihuela 2014). Some prevalent serotypes are reported to be good biofilm producers, and this may explain their high prevalence (Domenech et al. 2014; Domenech et al. 2015). Apparently, the chemical composition and structure of the capsular polysaccharide are important for the biofilm forming ability of different serotypes.

As discussed above, it is well established that *S. pneumoniae* colonization of the nasopharynx precedes the development of diseases such as OM (Hoa, Syamal, et al. 2009; Hoa, Tomovic, et al. 2009). Additionally, biofilm communities in the nasopharynx are a major source for horizontal genetic transference between pneumococcal strains (Marks et al. 2012; Wei and Håvarstein 2012). The sessile mode of living of *S. pneumoniae* is also advantageous with regards to evading the complement system and phagocytosis by human neutrophils (Domenech, Ramos-Sevillano, et al. 2013). Furthermore, cells released from nasopharyngeal biofilms can migrate to the ME where they will cause infection. Signals that induce dispersal of biofilms attached to epithelial cells, following an influenza A viral infection, include febrile-range temperatures, norepinephrine, extracytoplasmic ATP, and increased nutrient availability. These biofilm-released cells have phenotypic properties distinct from both biofilm and planktonic bacteria, with an increased capacity to disseminate and cause infection (Marks et al. 2013).

Another concern regarding *S. pneumoniae* in biofilms is its viability over extended periods, retaining their infection capacity (Marks et al. 2014). Secretions containing *S. pneumoniae* biofilms may be transferred via fomites from person to person and cause infection, which is very common to occur between children with OM attending day care centers (Marks et al. 2014).

The involvement of QS in the regulation of *S. pneumoniae* biofilm formation has also been studied. The effects of *luxS* expression and iron (in the form of Fe(III)) in biofilm development were found to be directly linked (Trappetti, Potter, et al. 2011). An overexpressing *luxS* mutant formed biofilms and supplementation with iron further enhanced biofilm formation. However, a *luxS*-defective mutant did not form biofilms even after iron supplementation. The *luxS*-dependent QS system is also a key regulator of early biofilm formation by *S. pneumoniae* (Vidal et al. 2011). These authors reported the construction of two *luxS* mutants that were unable to produce early biofilms, but this was reverted by genetic or physical complementation. Furthermore, transcription of *lytA* and *ply* (encoding the pneumococcal autolysin and pneumolysin, respectively) was found to be regulated by *luxS* (Vidal et al. 2011), and the role of Ply in early pneumococcal biofilm formation established (Shak et al. 2013). The LuxS/autoinducer 2 (AI-2) QS system, in which AI-2 determined by *luxS* accumulates in the external milieu and stimulates planktonic bacteria to initiate early biofilm formation, was also shown essential for biofilm formation and colonization of the ME epithelium by *S. pneumoniae* (Yadav et al. 2018). *S. pneumoniae* biofilms are also regulated by the Com QS system known for mediating the genetic transference between cells. The induction of the Com system by the competence stimulating peptide (CSP), the product of *comC* gene, promotes biofilm formation *in vitro* (Oggioni et al. 2006), though its impacts depend on the biofilm model used (Trappetti, Gualdi, et al. 2011; Vidal et al. 2013). Expression of *briC*, which is induced by the competence system, was also shown to have an impact on late stages of biofilm formation, increasing biofilm biomass and thickness (Aggarwal et al. 2018). Recently, the role of the Rgg/small hydrophobic peptide (SHP) QS system in *S. pneumoniae* biofilms was pointed as responsible for the regulation of exopolysaccharide synthesis, and its overexpression resulted in the production of a ticker polysaccharide capsule and reduced biofilm formation (Junges et al. 2017).

Alike *H. influenzae*, *S. pneumoniae* biofilms are less susceptible to antibiotics than planktonic growing bacteria (del Prado et al. 2010; Matejka et al. 2012). The slower growth of bacterial cells, the presence of

persister cells within the biofilm, the impaired antibiotic penetration through the biofilm matrix, among other factors, can affect the action of antibiotics (Takei et al. 2013). Despite the vast progress studying the specific role of biofilm cells and biofilm-released cells, as well as their interaction with the host during infection state there are still many questions that remain unanswered and need a deeper investigation.

Moraxella catarrhalis

Studies assessing the biofilm phenotype of *M. catarrhalis* are scarce. Despite this limited information, biofilm formation has been demonstrated *in vitro*, and biofilms have been detected in ME mucosal specimens of children with COME (Hall-Stoodley et al. 2006), and within adenoid biofilms of children with RAOM (Hoa, Tomovic, et al. 2009) (Table 2).

Addressing specifically *M. catarrhalis* biofilm formation (Supplemental Table S3), studies showed that UspA1 (Pearson et al. 2006), UspA2 (Verhaegh et al. 2008) and UspA2H proteins (Pearson and Hansen 2007), produced by only some *M. catarrhalis* strains, promote biofilm formation, while Hag protein inhibit biofilm formation (Pearson et al. 2006). Furthermore, *M. catarrhalis* isolates from children proved to have increased ability to form biofilms compared to adult isolates (Verhaegh et al. 2008).

Type IV pili play a role in *M. catarrhalis* biofilm formation, and mutants defective in type IV pili have impaired initial attachment, delayed microcolony formation and, diminished three-dimensional expansion (Luke et al. 2007). These authors showed multicellular mushroom-shaped structures separated by water channels in wild type strain biofilms and significantly less-robust biofilm when mutants were used (Luke et al. 2007).

The genetic expression of *M. catarrhalis* planktonic and biofilm cells, analyzed using DNA microarrays and reverse transcriptase PCR, showed that genes associated with energy generation, i.e. genes involved in nitrate, nitrite, and nitric oxide reduction, are upregulated in the biofilm state (Wang et al. 2007). Investigating the Host factor 1 (Hfq) protein, which function as an RNA chaperone that allows RNA-RNA interactions, in *M. catarrhalis* biofilm formation, it was observed that *hfq* mutants exhibited a growth advantage possibly due to the increased expression or relative abundance of several outer membrane proteins (Attia et al. 2008). The extracellular nuclease NucM encoded by *M. catarrhalis* was shown to affect cell aggregation and biofilm formation, with *nucM* mutants biofilms having an increased biomass compared with wild type strain biofilms (Tan et al. 2019).

These works provide evidence that *M. catarrhalis* forms biofilms, nevertheless, a more comprehensive understanding of the factors triggering biofilm formation and dispersal and their involvement in OM is desirable.

Multispecies

Epidemiological studies demonstrate that OM infections can be polymicrobial in nature (Post 1995; Matar et al. 1998; Gok et al. 2001; Hall-Stoodley et al. 2006; Hoa, Tomovic, et al. 2009; Aly et al. 2012; Holder et al. 2015; Cilveti et al. 2017), however, the interspecies interaction has been addressed only in a few studies (Figure 4 and Supplemental Table S4).

A study using the chinchilla infection model was used to evaluate the impact of *H. influenzae* and *S. pneumoniae* coinfection (Weimer et al. 2010). The results showed that, using a ratio of H. influenzae to *S. pneumoniae* of either 1:1 or 10:1, 89% *versus* 50% of the ears of mixed and single infection animals contained biofilms. It has also been reported that the contact of NTHi and pneumococcus with epithelial cells leads to an increased multispecies biofilm formation (Krishnamurthy and Kyd 2014), and NTHi can even provide passive protection to *S. pneumoniae* from killing by amoxicillin (Murrah et al. 2015). *H. influenzae* and *S. pneumoniae* showed alterations in gene expression when in co-culture, but expression levels were also dependent of the growth environment with genes encoding lactose and glycerol utilization, and sugar transport proteins differing in the levels expressed (Tikhomirova et al. 2015). The biofilm forming ability of *H. influenzae* and *S. pneumoniae* strains isolated from the nasopharynx of children with AOM revealed that almost 65% of *H. influenzae* and 67% of *S. pneumoniae* isolates produced biofilms (Vermee et al. 2019). Interestingly, better *H. influenzae* biofilm-producing strains were, in general, isolated from samples containing also *S. pneumoniae* but this correlation was not valid for *S. pneumoniae* strains.

H. influenzae and *M. catarrhalis* polymicrobial biofilms have an increased resistance towards antibiotics and host clearance compared to monospecies biofilms in chinchilla model of infection due to an interspecies QS process, in which *M. catarrhalis* uses AI-2 produced by *H. influenzae* to establish a persistent infection (Armbruster et al. 2010).

Multispecies biofilms by *S. pneumoniae* and *M. catarrhalis* showed that *M. catarrhalis* beta-lactamase production provided passive protection to *S. pneumoniae* against amoxicillin, and that *S. pneumoniae* increased the resistance of *M. catarrhalis* to azithromycin (Perez et al. 2014), with QS AI-2 having an important role in
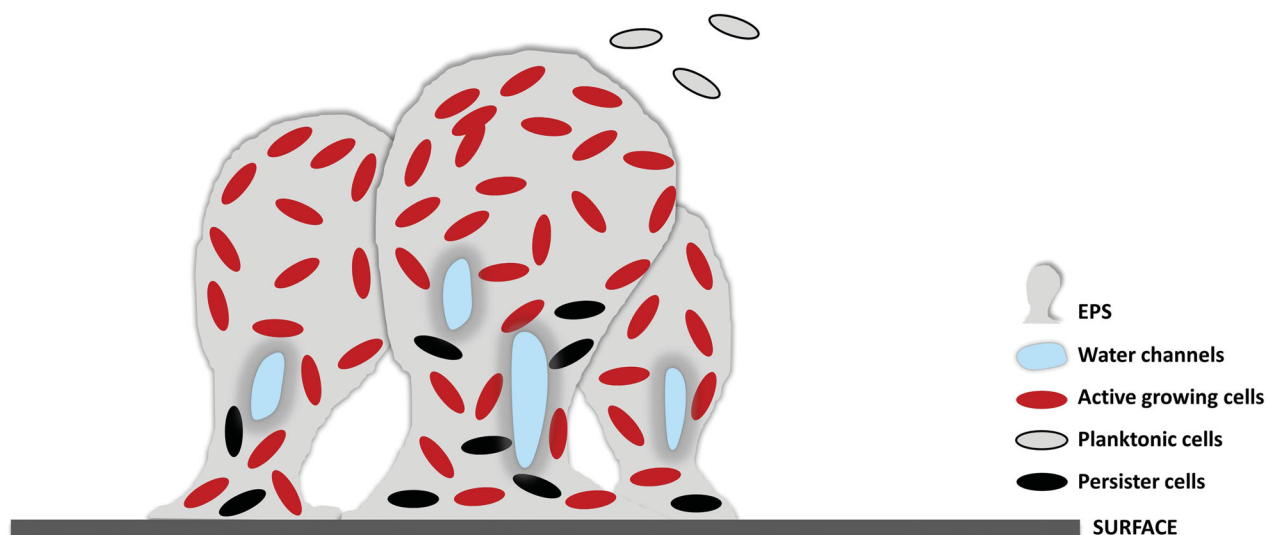
Figure 3. Representation of a mature biofilm.

Figure 4. Resume of the main findings of studies addressing multispecies biofilms in OM.

nasopharyngeal colonization and ME ascension during co-infections with both species (Perez et al. 2014).

Recently, the role of a less common OM pathogen, *Alloiococcus otitidis,* and its ability to form single and polymicrobial biofilms was investigated (Chan et al. 2017). *A. otitidis* is frequently detected by PCR in ME fluid samples, and multispecies biofilms formed together with *H. influenzae* resulted in a decreased antimicrobial susceptibility and promoted the survival of the latter species by increasing biofilm production in adverse conditions, such as depleted media and suboptimal growth temperature.

Treatment of otitis media

Current guidelines for the therapeutic management of ME infections depend on the type of OM that is diagnosed and are discussed below.

Management of symptoms such as ear pain and fever in AOM is made with analgesics, but the gold standard of treatment is, undoubtedly, the use of antibiotics (Figure 5). The majority of AOM cases resolve spontaneously and thus watchful waiting without immediate antibiotic prescription is recommended for nonsevere unilateral AOM in children 6 months to 23 months of age, and unilateral or bilateral nonsevere AOM in children with 24 months of age or older (Siddiq and Grainger 2015). In these cases, children must be followed up and antibiotherapy initiated if the symptoms do not improve in 48–72 h. Children with 6 or more months of age having a diagnosis of unilateral or bilateral AOM together with manifestation of severe signs or symptoms require immediate antibiotic prescription (Siddiq and Grainger 2015). In addition, antibiotics should be used for the treatment of nonsevere bilateral AOM in children 6 months through 23 months of age
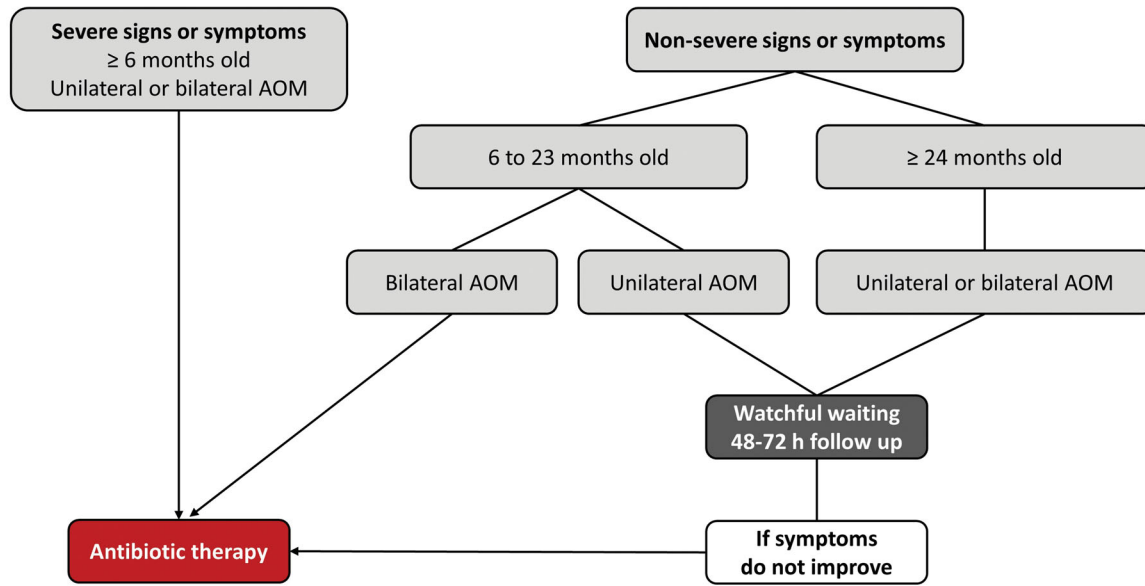
TREATMENT OF AOM

Figure 5. Guidelines for the treatment of AOM in children 6 months through 12 years of age.

(Siddiq and Grainger 2015). Amoxicillin at 80–90 mg/kg/day divided in two or three doses, is the drug of choice due to its low cost, acceptable taste, safety, effectiveness, and a narrow microbiologic spectrum (Harmes et al. 2013). Children having taken amoxicillin in the past 30 days, having conjunctivitis, or those needing coverage for β-lactamase-positive organisms (some *H. influenzae* isolates and universally all *M. catarrhalis*), should be prescribed with amoxicillin/clavulanic acid. Cephalosporins may be used in children presenting allergy to penicillin or in those that did not respond to the previous therapies. The use of nasal decongestants, antihistamines and corticosteroids is not recommended by the USA guidelines, since these have no apparent benefit (Siddiq and Grainger 2015).

For children with RAOM, myringotomy and insertion of a ventilation tube may be considered. In this surgery, a small incision is made in the tympanic membrane so that a tympanostomy tube can be inserted to enable ME fluid draining and improve its ventilation. Tympanostomy tubes usually stay in the tympanic membrane for 6 to 12 months and fall out themselves. Although a reduction in RAOM cases is observed with the prophylactic administration of antibiotics, these are not recommended due to their associated side effects (Harmes et al. 2013; Qureishi et al. 2014; Schilder et al. 2016).

In OME cases, a 3-month period of watchful waiting is recommended to evaluate the degree of hearing loss and its impact on speech, language and learning before treatment. According to the USA guidelines, besides decongestants, antihistamines and corticosteroids, antibiotics should also not be prescribed due to their low long-term outcome. When the ME effusion persists for more than 3 months (COME), myringotomy and ventilation tube insertion with or without adenoidectomy may be considered for the treatment of children (Harmes et al. 2013; Qureishi et al. 2014; Rosenfeld et al. 2016; Schilder et al. 2016).

Novel strategies targeting otitis media biofilms

The increasing antimicrobial resistance has forced a search for novel strategies to treat ME infections that focus on antibiofilm effects (Supplemental Table S5).

Disruption of the EPS, mostly by the interaction of compounds with the eDNA present within the *H. influenzae* biofilm matrix, is an example of antibiofilm focused research (Cavaliere et al. 2014). The main idea is to destabilize the EPS by targeting DNABII family of proteins, positioned at the vertices of crossed strands of eDNA. According to the authors, the use of a cation chelator (e.g. Ethylenediaminetetraacetic acid) was effective in both prevention and treatment of NTHi *in vitro* biofilms, and both EDTA and DNaseI enhanced the efficacy of ampicillin and ciprofloxacin against NTHi biofilms. Antibodies against the Integration Host Factor (IHF) member of the DNABII family showed rapid disruption of NTHi biofilms *in vitro* (Goodman et al. 2011). Furthermore, the transcutaneous immunization with purified IHF of chinchillas with pre-formed NTHi biofilms resulted in a rapid disease resolution, and IHF antisera acted synergistically with amoxicillin. The

mechanism of action of anti-IHF-mediated biofilm disruption is based on the sequestration of free IHF upon its dissociation from the eDNA matrix, forcing an equilibrium shift and ultimately, collapse of the biofilm (Brockson et al. 2014). Besides polyclonal antibodies action to disrupt NTHi biofilms, a cocktail of monoclonal antibodies directed against specific epitopes of the DNABII protein IHF was used to disrupt NTHi biofilms in the ME of the chinchilla infection model (Novotny et al. 2016). The treated chinchillas showed a marked reduction or complete eradication of biofilms in the ME with little to no residual signs of inflammation.

Antibiofilm therapeutic approaches have also focused on outer membrane proteins and adhesins expressed by NTHi, such as type IV pili (TFP) and OMP P5. Transcutaneous immunization with TFP and P5-targeted immunogens was effective in both prevention and treatment of NTHi biofilms in the ME of chinchillas (Novotny et al. 2011; Novotny, Clements, et al. 2015). Antibodies directed against TFP mediated gradual "top-down" dispersal of NTHi from the biofilms. In addition, dispersal was both TFP expression and LuxS AI-2 QS signaling molecules dependent (Novotny, Jurcisek, et al. 2015).

Natural and synthetic compounds have also been investigated for the control of biofilms formed by *H. influenzae*. For instance, pyrazol derivatives (Kosikowska et al. 2014), chalcones (Kunthalert et al. 2014), plant extracts (Wajima et al. 2016), nitric oxide (Collins et al. 2016), proteins (Dawe et al. 2017), and honey (Newby et al. 2018), among others, have shown positive effects.

Antibiofilm experiments targeting *S. pneumoniae* have also been addressed. For instance, bacterial autolysin LytA and the and phage lysozymes Cpl-1 and Cpl-7 reduced around 80%, 70% and 55%, respectively (Domenech et al. 2011). Other proposed alternative or adjuvant antimicrobial therapies to fight pneumococcal biofilms include xylitol (Kurola et al. 2011), HAMLET (a natural complex from human milk) (Laura R. Marks et al. 2012), plant extracts or essential oils (Yadav et al. 2013; Talekar et al. 2014; Minami et al. 2017), ceragenins (Moscoso et al. 2014), nitric oxide (Allan et al. 2016; Allan et al. 2017), zinc oxide nanoparticles (Bhattacharyya et al. 2018), flavonoids (Wang et al. 2018), and even human amniotic/chorionic membrane extracts (Yadav et al. 2017), among others.

PCVs are available for use in infants, young children, elderly, and children and adults who are at increased risk for getting pneumococcal disease. The current vaccine, PCV13, provides protection against 13 pneumococcal serotypes. Besides this, there is also available a pneumococcal polysaccharide vaccine (PPSV23) that provides protection to 23 *S. pneumoniae* serotypes. However, this later vaccine is only recommended for elderly people and for children and adults older than 2 years that are at increased risk for disease (Centers for Disease Control and Prevention 2017). Widespread use of PCVs led to reductions in AOM episodes caused by vaccine-type *S. pneumoniae* serotypes, and it is hypothesized that this reduction can modify the continuum of otitis media pathogenesis, by decreasing the occurrence of early middle ear damage, subsequent OM episodes caused by non-vaccine pneumococcal serotypes as well as NTHi and *M. catarrhalis,* and also biofilm formation. Therefore, PCVs have an important role in the pathogenic progression of the disease when administered before the occurrence of any OM case. However, once initial damage occurs, PCVs cannot reduce disease beyond that caused by vaccine serotypes (Dagan et al. 2016).

M. catarrhalis antibiofilm strategies have not been extensively studied. Nevertheless, hyaluronic acid was found to reduce poorly (about 30%) *M. catarrhalis* biofilms (Drago et al. 2014), while photodynamic therapy with porfimer sodium reduced by 3–4 logarithmic units the viable bacteria present in biofilms (Luke-Marshall et al. 2014).

Treatment of multispecies biofilms has been reported using antioxidant compounds, particularly N-acetyl-L-cysteine and cysteamine (Domenech and García 2016), esters of bicyclic amines (Roig-Molina et al. 2019), and also antibodies against TFP of NTHi prevented and disrupted multispecies biofilms via an interspecies quorum signal at temperatures that mimic the nasopharynx (34 °C) or the ME (37 °C) (Mokrzan et al. 2018).

Although some of the described approaches seem to be promising for the control of biofilms formed by otopathogens, to our knowledge none are currently undergoing clinical trials, and therefore, their potential clinical use might still be afar.

Concluding remarks

Investigations over the last years have proven that biofilms are associated with OM, contributing to its recurrence and chronicity. Understanding which factors influence biofilm formation and dispersal by the most common otopathogens as well as how they interact with each other and with the host enable the development of novel and targeted-therapeutic agents.

Disclosure statement

No potential conflict of interest was reported by the authors.

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