ORIGINAL ARTICLE

The prevalence of middle ear pathogens in the outer ear canal and the nasopharyngeal cavity of healthy young adults

T. De Baere[1,3], M. Vaneechoutte[1], P. Deschaght[1], J. Huyghe[2] and I. Dhooge[2]

1) Laboratory Bacteriology Research, 2) Department of Oto-rhino-laryngology, University Hospital Ghent, Gent and 3) Department of Bacteriology, Scientific Institute of Public Health, Brussels, Belgium

Abstract

Culturing middle ear fluid samples from children with chronic otitis media with effusion (OME) using standard techniques results in the isolation of bacterial species in approximately 30–50% of the cases. *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, the classic middle ear pathogens of acute otitis media, are involved but, recently, several studies suggested *Alloiococcus otitidis* as an additional pathogen. In the present study, we used species-specific PCRs to establish the prevalence, in both the nasopharyngeal cavity and the outer ear, of *H. influenzae*, *M. catarrhalis*, *S. pneumoniae* and *A. otitidis*. The study group consisted of 70 healthy volunteers (aged 19–22 years). The results indicate a high prevalence (>80%) of *A. otitidis* in the outer ear in contrast to its absence in the nasopharynx. *H. influenzae* was found in both the outer ear and the nasopharynx (6% and 14%, respectively), whereas *S. pneumoniae* and *M. catarrhalis* were found only in the nasopharynx (9% and 34%, respectively). *A. otitidis*, described as a fastidious organism, were able to be cultured using an optimized culture protocol, with prolonged incubation, which allowed the isolation of *A. otitidis* in five of the nine PCR-positive samples out of the total of ten samples tested. Given the absence of the outer ear inhabitant *A. otitidis* from the nasopharynx, its role in the aetiology of OME remains ambiguous because middle ear infecting organisms are considered to invade the middle ear from the nasopharynx through the Eustachian tube.

Keywords: Detection, middle ear pathogens, otitis media
Original Submission: 7 November 2008; **Revised Submission:** 13 February 2009; **Accepted:** 13 February 2009
Editor: F. Allerberger
Article published online: 6 November 2009
Clin Microbiol Infect 2010; **16**: 1031–1035
10.1111/j.1469-0691.2009.02928.x

Corresponding author and reprint requests: T. De Baere, Department of Bacteriology, Scientific Institute of Public Health, Brussels, Belgium
E-mail: Thierry.Debaere@iph.fgov.be

Introduction

Otitis media with effusion (OME) is a frequently diagnosed disease in infants and young children and it is the most common illness for which children receive antibiotics before the age of 10 years [1,2]. The three major middle ear pathogens are *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* [2]. However, during recent years, several studies were published suggesting that *Alloiococcus otitidis* was an additional pathogen [3–6], and was detected at a higher rate in the middle ear than the three major pathogens. In agreement with these studies, we were able to demonstrate a high prevalence of *A. otitidis* in middle ear fluid [7]. To further elucidate the possible pathological role of *A. otitidis*, we investigated its presence in the nasopharynx and outer ear of a group of healthy young adults.

The detection of *A. otitidis* is mostly PCR-based because it is a difficult to culture the organism, and most previous studies of *A. otitidis* and OME have presented PCR-positive and culture-negative results. Accordingly, we adapted a previously reported culture protocol [8] to allow the isolation of *A. otitidis* from clinical samples.

Materials and methods

Patient population and sampling methods

The study group consisted of 70 healthy volunteers, who were not suffering from ear or throat problems at, or shortly before, the moment of sampling. The patients were aged 19–22 years. Two different samples were taken. External ear canal swabs were collected by a gentle swabbing of the exter-

nal canal through a speculum, thus avoiding contact with the auricle and meatus and a pernasally taken nasopharyngeal swab. Both swabs were sent to the microbiology laboratory immediately and processed for DNA extraction and culture. Because the optimized culture protocol (see below) was initially not available, only ten of the swabs were cultured for *A. otitidis* according to this protocol.

All participants provided their written informed consent. The study was conducted in accordance with the ethical standard stipulated in the Helsinki declaration (2000) for research involving human subjects.

Sample processing

The dry swabs were swirled in 300 μL of physiological water (NaCl 0.7%). Two hundred microlitres of the suspension were transferred to 800 μL of NucliSENS Easymag Lysis buffer (Bio-Mérieux, Marcy-l'Etoile, France) and homogenized by vortexing. After an incubation period of 10 min at room temperature, the samples were stored at −80°C until further processing. The DNA extraction was carried out in batches of 24 samples on the automated DNA extractor NucliSENS EasyMag (Bio-Mérieux), which resulted in 110 μL of eluate (i.e. DNA extract).

Species-specific PCR for the detection of middle ear pathogens

The DNA extracts were subjected to species-specific PCRs for *A. otitidis*, *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* [9] and *Pseudomonas aeruginosa* with a major modification: the PCR for the detection of the four species [9] was not carried out as a multiplex PCR, but in four separate amplification reactions, because we were unable to reach sufficient sensitivity with the multiplex format in preliminary experiments.

Culture

Culture was carried out for a subset of ten outer ear samples. Fifty microlitres of the mixture obtained by swirling the swab in physiological water for DNA extraction were used to inoculate a blood agar plate, consisting of Tryptic Soy Agar containing sheep blood (5%) (Becton Dickinson, Erembodegem, Belgium), which was incubated aerobically at 37°C for up to 5 days. Small α-haemolytic colonies were transferred to a heart infusion agar containing sheep blood (5%) (Becton Dickinson) for subculturing aerobically at 37°C for 3–5 days.

Identification of cultured organisms

Colonies were identified using tRNA intergenic spacer length polymorphism analysis (tDNA-PCR), according to a previously described protocol [10]. Briefly, the intergenic spacers between different tRNA genes were amplified and separated using capillary electrophoresis on an ABI310 apparatus (Applied BioSystems, Foster City, CA, USA). The resulting digitized fingerprint was compared with the fingerprints available in a large database obtained from reference and well-identified strains, allowing species identification. For the identification of *A. otitidis*, the reference fingerprints were prepared using two culture collection strains (Culture Collection, University of Göteborg, Sweden [CCUG] 33997 and CCUG 44657), resulting in a species-specific fingerprint composed of tRNA intergenic fragment lengths of 57, 59.9, 66.5, 67.9, 149, 151.4, 152.2, 161 and 249.7 bp, which were not observed for any other of the hundreds of species already contained in the tDNA-PCR library.

In addition, two of the five *A. otitidis* isolates, and some isolates for which the tDNA-PCR identification was not conclusive, were identified using 16S rRNA gene sequencing as described previously [11]. Comparison of the obtained sequences with a selection of high-quality sequences was carried out using the SmartGene, IDNS software (Zug, Switzerland).

Results

Prevalence of the middle ear pathogens

The results obtained for the species-specific PCRs are presented in Table 1. Fifty-eight out of 70 outer ear samples were positive for *A. otitidis*, which was the most frequently detected organism in the outer ear canal. This contrasts with the detection rate in nasopharyngeal samples, none of which tested positive for *A. otitidis*.

H. influenzae was the only organism of the three known middle ear pathogens that was detected in the outer ear (6%). The rates of detection of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* in the nasopharyngeal cavity were 14%, 9% and 34%, respectively. None of the subjects tested positive for *H. influenzae* from the nasopharynx and outer ears.

Culture

We were able to increase the culture efficiency for *A. otitidis* by prolongation of the initial period of aerobic incubation on blood agar from which α-haemolytic small colonies were

TABLE 1. Percentage of positive PCR results for 70 healthy young adults

Species	Outer ear canal	Nasopharynx
Alloicococcus otitidis	83	0
Haemophilus influenzae	6	14
Moraxella catarrhalis	0	34
Streptococcus pneumoniae	0	9

picked after 5 days and then aerobically subcultured on heart infusion agar containing sheep blood (5%) (Becton Dickinson), aiming to minimize the chance that the small *A. otitidis* colonies were overgrown by other organisms present in the sample.

An optimized culture method was tested on a subset of ten outer ear samples (i.e. two sets of five samples on separate testing days). The results are presented in Table 2. Of these randomly chosen samples, nine were positive for *A. otitidis* by PCR, which correlates well with the overall PCR positivity of 83% in the present study. The isolation of *A. otitidis* was obtained from five of the tested samples, resulting in a culture positivity of 50%, or even 55% of the PCR-positive samples (5/9). The only sample that was negative by PCR was also found to be negative by culture.

Only one of all ten culture results presented a pure culture (Table 2, subject L.70); all other samples yielded cultures of different species in one sample, with *Turicella otitidis*, *Staphylococcus capitis*, *Staphylococcus auricularis* and *Corynebacterium auris* being isolated from more than one subject (Table 2).

TABLE 2. Results obtained by *Alloicoccus otitidis*-PCR and culture for the outer ear swabs of the subsets of ten patients

Subject	*Alloicoccus otitidis* PCR	*Alloicoccus otitidis*	*Brevibacterium* sp.	*Corynebacterium auris*	*Micrococcus lylae*	*Micrococcus luteus*	*Staphylococcus auricularis*	*Staphylococcus capitis*	*Staphylococcus epidermidis*	*Staphylococcus intermedius*	*Turicella otitidis*
L.47	+			+							+
L.48	+						+				+
L.49	+				+						+
L.50	+	+									+
L.51	+	+				+					
L.67	+	+						+		+	
L.68	+	+					+				+
L.69	+							+	+		
L.70	+	+		+				+			
L.66	–		+								

Discussion

OME is an inflammation of the middle ear space resulting in a collection of fluid behind an intact tympanic membrane. Hearing loss is the main clinical feature. The pathophysiological mechanisms resulting in the persistence of OME are not well understood. Although dysfunction of the Eustachian tube is an important factor, there is increasing interest in the role of persistent bacterial infection and associated chronic inflammation as the cause of chronic OME. Using standard culture techniques, bacterial species have been isolated in approximately 30–50% of cases of middle ear effusions in chronic OME, with the majority comprising the classical middle ear pathogens.[12].

In the present study, we used species-specific PCR to determine the prevalence in both the outer ear and the nasopharyngeal cavity of healthy subjects of the three classical middle ear pathogens and of *A. otitidis*, which has been considered by several groups as a possible middle ear pathogen [3,4,6,13]. As expected, the commonly known otitis media pathogens were detected in the nasopharyngeal cavity. Only *H. influenzae* was also detected in the outer ear. By contrast, *A. otitidis* was very prevalent in the outer ear canal (83% of 70 subjects), but was completely absent in the nasopharynx, which is generally accepted to be the reservoir of middle ear pathogens. Table 3 summarizes the results of previous studies with regard to the presence of *A. otitidis* in the outer ear and nasopharynx.

TABLE 3. Prevalence of *Alloiococcus otitidis* in outer ears and/or nasopharynx, as reported in previous studies

Study	Sample type	Number and type of patients	Age[a]	Species studied	Prevalence (%) of *Alloicococcus otitidis*	
					Culture	PCR
[17]	External ear swab	147 healthy idividuals	Adults + children	All bacteria	14	NA
	Cerumen swab	148 healthy individuals		All bacteria	19	NA
[15]	Outer ear	1119 children[b]	Children (9 months–14 years)	*A. otitidis*	2	NA
[18]	Outer ear	24 healthy individuals	Adults + children	All bacteria	NA	85
[13]	Nasopharyngal swab	56 OME	Children	*A. otitidis*	0	11
	Middle ear effusion fluid	83 OME	(8 months–10 year)	*A. otitidis*	0	29

NA, not applied.
[a]Age is given as adults and/or children. The age range of the patient group is added, when available.
[b]The patient group consisted of hospitalized patients and outpatients, without specification on whether all or only part were patients with OME.
OME, otitis media with effusion.

During the present study, an optimized culture protocol was developed, combining previously published data. A recent study [8] used a wide variety of solid culture media in combination with pre-incubation enrichment in brain heart infusion broth. The overall conclusion was that a prolonged culture period is needed. These authors cultured for *A. otitidis* in the presence of 7.5% CO_2, which is an atmospheric condition shown to be disadvantageous for the culture of this organism [14]. Some studies recommend the use of sheep blood, chocolate agar or brain heart infusion agar containing rabbit blood (5%) [15,16]. The conclusion of a detailed comparison of the growth requirements of *A. otitidis* [14] revealed that the best results were obtained using heart infusion agar containing rabbit blood (5%) with aerobic incubation at 37°C. The present study, comparing and combining these findings, resulted in the following optimized method: the samples were inoculated on Tryptic Soy Agar containing sheep blood (5%) (Becton Dickinson) and incubated aerobically at 37°C during a prolonged period of up to 5 days. Because the small colonies of *A. otitidis* may be overgrown during prolonged culture, small α-haemolytic colonies were picked and subcultured aerobically on heart infusion agar containing sheep blood (5%) (Becton Dickinson), resulting in clearly visible α-haemolytic colonies within 3–5 days. Because the culture conditions were not yet optimized at the start of this study, only a subset of ten samples was tested. The optimized culture protocol enabled us to detect *A. otitidis* in five of these samples, which is less efficient than PCR-based detection (nine samples positive for *A. otitidis*) but significantly better than the results obtained in previous studies using shorter culture protocols (Table 3).

The use of a general culture medium also allowed culture of other organisms present in the healthy outer ear. We were able to isolate, for example, *T. otitidis* (n = 5), *S. capitis* (n = 3), *S. auricularis* (n = 2), *C. auris* (n = 2) and some other *Staphylococcus* and *Micrococcus* species (Table 2).

Two studies, both of patient groups of adults and children, reported previously on the composition of the microflora of the healthy outer ear. First, a culture-based study [17] reported that the two most common groups of organisms isolated from the external ear canal were *Staphylococcus* species, predominantly *S. auricularis* (20%), *Staphylococcus epidermidis* (11%) and *S. capitis* (10%), and coryneforms, i.e. *C. auris* (2%) and the related *T. otitidis* (12%), followed by *A. otitidis* (6%). Another study, using universal bacterial PCR, in combination with cloning and sequencing [18], reported 56% of the clones from the outer ear canal as *A. otitidis*, 20% as '*Corynebacterium otitidis*' and 10% as *S. auricularis*. '*C. otitidis*' is, however, not a validated species and turns out to be synonymous with *T. otitidis*, because the only entry for '*C. otitidis*' in the 16S rRNA gene sequence GenBank database (i.e. X73976) corresponds to that of the type strain of *T. otitidis* [19]. Taking into account the use of a suboptimal *A. otitidis* culture method in the study of Stroman *et al.* [17], it can be concluded that *A. otitidis* is probably the most common organism in the healthy outer ear, in the narrow age group of 19–22 years of this study and in the overall population of both adults and children. Other common organisms are *T. otitidis* and members of the genera *Staphylococcus* and *Corynebacterium*.

Two other culture-based studies reported high isolation rates for *A. otitidis*, but from middle ear effusion (i.e. in 40% of 50 children referred for myringotomy [8] and in 48% of 110 OME patients aged between 1 and 12 years [20]), whereas most studies found between 0% and 5% of *A. otitidis* in OME patients [8].

In conclusion, *A. otitidis* is one of the most frequently occurring commensal inhabitants of the outer ear which is completely absent from the nasopharynx. Its presence in approximately half of the middle ear infections, as reported by some recent studies [8,20], is most probably best explained by secondary colonization of the middle ear, which

A. otitidis enters through (temporarily) opened ear drums as a consequence of middle ear infection due to other bacteria. Indeed, de Miguel Martinez and Macias [20] found *A. otitidis* to be completely absent from closed ears in children with acute otitis media. Furthermore, in our previous study [7], the high incidence of *A. otitidis* was predominantly observed in a subgroup of patients with an open ear drum. In the non-OME patients with other ear problems, but with fully closed eardrums, the incidence of *A. otitidis* was much lower. Therefore, the high incidence of *A. otitidis* in middle ear fluid of OME patients is most likely not related to a pathogenic role of this organism in this condition and is probably best explained by either secondary invasion from the outer ear to the middle ear, after (transient) opening of the ear drum or by contamination with commensal bacteria present in the outer ear during the sampling process.

Acknowledgements

Part of this study was presented at the 18th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 2008 (abstract P1968).

Transparency Declaration

T. De Baere is indebted to the FWO (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen) for a postdoctoral fellowship. All authors declare that there is no presence of dual or conflicting interest in this article.

References

1. Klein JO. Management of otitis media: 2000 and beyond. *Pediatr Infect Dis J* 2000; 19: 383–387.
2. Bluestone CD, Stephenson JS, Martin LM. Ten-year review of otitis media pathogens. *Pediatr Infect Dis J* 1992; 11: S7–S11.
3. Harimaya A, Takada R, Hendolin PH, Fujii N, Ylikoski J, Himi T. High incidence of *Alloiococcus otitidis* in children with otitis media, despite treatment with antibiotics. *J Clin Microbiol* 2006; 44: 946–949.
4. Hendolin PH, Karkkainen U, Himi T, Markkanen A, Ylikoski J. High incidence of *Alloiococcus otitis* in otitis media with effusion. *Pediatr Infect Dis J* 1999; 18: 860–865.
5. Kalcioglu MT, Oncel S, Durmaz R, Otlu B, Miman MC, Ozturan O. Bacterial etiology of otitis media with effusion; focusing on the high positivity of *Alloiococcus otitidis*. *New Microbiol* 2002; 25: 31–35.
6. Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. The clinical role of *Alloiococcus otitidis* in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002; 66: 41–48.
7. De Baere T, Hollants J, Waeytens A *et al.* Otitis media microbes: Culture, PCR and confocal laser scanning microscopy. *B-Ent* 2009; 5: 65–72.
8. Ashhurst-Smith C, Hall ST, Walker P, Stuart J, Hansbro PM, Blackwell CC. Isolation of *Alloiococcus otitidis* from indigenous and non-indigenous Australian children with chronic otitis media with effusion. *FEMS Immunol Med Microbiol* 2007; 51: 163–170.
9. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J Clin Microbiol* 1997; 35: 2854–2858.
10. Baele M, Storms V, Haesebrouck F *et al.* Application and evaluation of the interlaboratory reproducibility of trna intergenic length polymorphism analysis (tDNA-PCR) for identification of *Streptococcus* species. *J Clin Microbiol* 2001; 39: 1436–1442.
11. van Kempen M, Dhooge I, Vaneechoutte M, Claeys G. Turnover of *Haemophilus influenzae* isolates in otitis-prone children. *Acta Otorhinolaryngol Belg* 2001; 55: 83–86.
12. Dhooge I, Desloovere C, Boudewyns A, Van Kempen M, Dachy JP. Management of otitis media with effusion in children. *B-Ent.* 2005; Suppl 1: 3–13; quiz 14–15.
13. Harimaya A, Takada R, Somekawa Y, Fujii N, Himi T. High frequency of *Alloiococcus otitidis* in the nasopharynx and in the middle ear cavity of otitis-prone children. *Int J Pediatr Otorhinolaryngol* 2006; 70: 1009–1014.
14. Miller PH, Facklam RR, Miller JM. Atmospheric growth requirements for *Alloiococcus* species and related gram-positive cocci. *J Clin Microbiol* 1996; 34: 1027–1028.
15. Gomez-Hernando C, Toro C, Gutierrez M, Enriquez A, Baquero M. Isolation of *Alloiococcus otitidis* from the external ear in children. *Eur J Clin Microbiol Infect Dis* 1999; 18: 69–70.
16. Bosley GS, Whitney AM, Pruckler JM *et al.* Characterization of ear fluid isolates of *Alloiococcus otitidis* from patients with recurrent otitis media. *J Clin Microbiol* 1995; 33: 2876–2880.
17. Stroman DW, Roland PS, Dohar J, Burt W. Microbiology of normal external auditory canal. *Laryngoscope* 2001; 111: 2054–2059.
18. Frank DN, Spiegelman GB, Davis W, Wagner E, Lyons E, Pace NR. Culture-independent molecular analysis of microbial constituents of the healthy human outer ear. *J Clin Microbiol* 2003; 41: 295–303.
19. Funke G, Stubbs S, Altwegg M, Carlotti A, Collins MD. *Turicella otitidis* gen. Nov., sp. Nov., a coryneform bacterium isolated from patients with otitis media. *Int J Syst Bacteriol* 1994; 44: 270–273.
20. de Miguel Martinez I, Macias AR. Serous otitis media in children: implication of *Alloiococcus otitidis*. *Otol Neurotol* 2008; 29: 526–530.