The Journal of Laryngology & Otology
November 2005, Vol. **119,** pp. 862–865

Impact of antibiotics on pathogens associated with otitis media with effusion

YUKIKO HAMAMOTO, MD, YUKAKO GOTOH*, MD, YOSHIMI NAKAJO*, MD, SATOKO SHIMOYA*, MD, CHIKAKO KAYAMA*, MD, SHINGO HASEGAWA*, MD, KEN-ICHI NIBU, MD

Abstract

Objective: To further understand the roles of bacteria and antibiotics in the development of otitis media with effusion (OME).

Methods: Samples of middle-ear effusion (MEE) were collected during the placement of ventilation tubes to treat chronic OME. Children with acute otitis media within the past three months were excluded from this study. We used polymerase chain reaction (PCR) to detect pathogens and to test the susceptibility of *Streptococcus pneumoniae* to penicillin.

Results: Among MEE samples from 52 children, PCR detected bacterial DNA in 32 per cent (24/75) of them. *S. pneumoniae* was detected more frequently in middle ears that required ventilation tube insertion at least twice compared with those requiring ventilation tube insertion only once (5/15 versus 4/60; $p = 0.013$). Higher levels of *S. pneumoniae* were detected in MEE from children with, than without, a long history of antibiotic administration (7/10 versus 2/14; $p = 0.0187$). The *pbp* genes of all isolated *S. pneumoniae* contained mutations.

Conclusions: Long exposure to antibiotics might significantly influence the bacterial genome in MEE.

Key words: Otitis Media With Effusion; Bacteria; Polymerase Chain Reaction; Antibiotics

Introduction

Otitis media with effusion (OME) is a widespread disease of childhood that causes a transient conductive hearing loss, which, when persistent, can cause delayed speech, language and social development. The suggested causative mechanisms of OME include infection, inflammation of the middle ear, dysfunctions of the Eustachian tube, and immune complexes or endotoxins in the middle ear. However, details of the mechanism of OME remain unknown.

Senturia[1] originally noted the presence of bacteria in middle-ear effusions (MEE) of OME. *Streptococcus pneumoniae, Moraxella catarrhalis and Haemophilus influenzae,* the most common pathogens in acute otitis media, have occasionally been found in MEE of OME.[2]

To further study the roles of these bacteria in the development of OME, we examined the MEE of children with chronic OME without an episode of acute otitis media within three months, by culturing bacteria and by polymerase chain reaction (PCR) amplification. We also examined the sensitivity of *S. pneumoniae* to penicillin by PCR and compared the results with the clinical course of OME.

Patients and methods

The MEE samples were collected while ventilation tubes were being placed under general anaesthesia to treat chronic OME at the Kohnan Hospital. At the time of myringotomy, MEE were aspirated into Tym-Tap collectors (Juhn Tym-Tap; Xomed Inc, Jacksonville, FL, USA). Each MEE sample was described as mucous, serous, or purulent. A portion of each MEE was sent to the bacterial laboratory where selective agars differentiated *S. pneumoniae, M. catarrhalis* and *H. influenzae.* The remainder of each sample was stored at –80°C. Clinical data were obtained from medical records. All protocols associated with this study were approved by the Ethical Committees of Kobe University Graduate School of Medicine and of the Kohnan Hospital. Written informed consent to participate in all procedures associated with this study was obtained from the parents of all of the children.

DNA purification and PCR

DNA was extracted from the MEE samples using Isogen-LS (Nippon Gene, Japan) according to the manufacturer's protocol. The PCR-based assays

From the Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kobe University and the *Kohnan General Hospital, Kobe, Japan.
Accepted for publication: 26 May 2005.

TABLE I
POLYMERASE CHAIN REACTION (PCR) PRIMERS

Primer	Direction	Sequence
S. pneumoniae	F	TGA AGC GGA TTA TCA CTG GC
	R	GCT AAA CTC CCT GTA TCA AGC G
H. influenzae	F	ACT TTT GGC GGT TAC TCT GT
	R	TGT GCC TAA TTT ACC AGC AT
M. catarrhalis	F	GTC GCA CGC CAA CAC TTG CT
	R	ATT GTC GTA TGA GCG GTA AT
β-actin	F	CCC ATG CCA TCC TGC GTC TG
	R	CGT CAT ACT CCT GCT TGC TG
pbp1a	F	AAA CAA GGT CGG ACT CAA CC
	R	AGG TGC TAC AAA TTG AGA GG
pbp2x	F	CCA GGT TCC ACT ATG AAA GTG
	R	CAT CCG TCA AAC CGA AAC GG
pbp2b	F	CAA TCT AGA GTC TGC TAT GGA
	R	GGT CAA TTC CTG TCG GCA GTA

F = forward; R = reverse. Primers for β-actin served as positive control. *Streptococcus pneumoniae* resistance was investigated using primers for pbp1a, pbp2x and pbp2b.

detected DNA of *S. pneumoniae,*[3] *H. influenzae* and *M. catarrhalis.*[2] Primers for β-actin served as positive controls. Table I shows the primers designed for β-actin, *S. pneumoniae, H. influenzae* and *M. catarrhalis.* Penicillin susceptibility was investigated using the primers for pbp1a, pbp2x and pbp2b.[4]

For PCR, 1.25 μl of template DNA was added to 23.75 μl of a reaction mixture containing 2.5 μl of 10x PCR buffer, 0.5 μl of 10 mM of a mixture of deoxynucleoside triphosphates, 0.75 μl of 50 mM $MgCl_2$, 0.75 μl of primer mixture (10 μM each), 0.125 μl of Taq polymerase (Life Technologies) and 19.125 μl of distilled water. The PCR cycling conditions consisted of: denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 45 sec, 60°C for 60 sec and 72°C for 90 sec. After the final cycle, the reaction mixtures were incubated at 72°C for 7 min. All reaction mixtures were analysed by electrophoresis in 2 per cent agarose gels.

Results

Between September 2001 and July 2004, ventilation tubes were inserted into 52 children at Kohnan Hospital, Kobe, Japan for at least three months to treat chronic OME. Children who developed acute otitis media within three months before tube insertion were not included in this study. Myringotomy was performed in 75 ears of 52 children from 40 boys and 12 girls (average age 4.7 years; range 20 months to 10 years). MEE samples were obtained from all these 75 ears. There were 64 mucous types and 11 serous types. Follow-up periods ranged from 6 to 36 months.

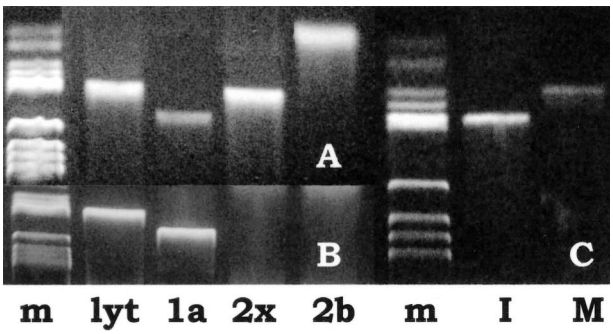

FIG. 1

Pathogens in middle-ear effusion (MEE) samples detected by polymerase chain reaction (PCR). *Streptococcus pneumoniae* in nasopharyngeal secretion (A). *S. pneumoniae* (B), *Haemophilus influenzae* and *Moraxella catarrhalis* (C) in MEE. *S. pneumoniae* in panel B was penicillin-resistant, since no PCR products of 2x and 2b genes were obtained. I = *H. influenzae*; M = *M. catarrhalis*; m = marker; lyt = *S. pneumoniae*.

Bacterial findings

All 75 MEE samples were cultured for bacteria and assayed by PCR (Figure 1). Bacterial culture detected *H. influenzae* and *M. catarrhalis* in four MEE samples each and no *S. pneumoniae* in any of them. PCR assays detected bacterial DNAs in 32 per cent (24/75) of MEE samples from 19 children. *S. pneumoniae, H. influenzae* and *M. catarrhalis* were detected in 9, 17 and 10 MEE samples, respectively. All pathogens detected by culture, were also detected by PCR. Figure 2 shows that two-thirds of the detected *H. influenzae* were present without other pathogens, whereas most *S. pneumoniae* and *M. catarrhalis* co-existed with other pathogens. Pathogens were detected in 31 per cent (20/64) of the mucous type and in 36 per cent (4/11) of the serous type of MEE.

S. pneumoniae *was frequently detected in the refractory ears*

We detected *S. pneumoniae* more frequently in OME that required ventilation tube insertion, at least twice than in that requiring insertion only once (5/15 versus 4/60; $p = 0.013$).

Impact of prolonged antibiotic exposure on pathogens in MEE samples

Of all 24 ears in which MEE bacterial DNA was detected by PCR, antibiotics had been administered for over one month to treat ear and/or nasal diseases at other out-patient clinics before the patients were referred to our hospital. The frequency of *S. pneumoniae* was significantly higher in MEE

https://doi.org/10.1258/002221505774783476 Published online by Cambridge University Press

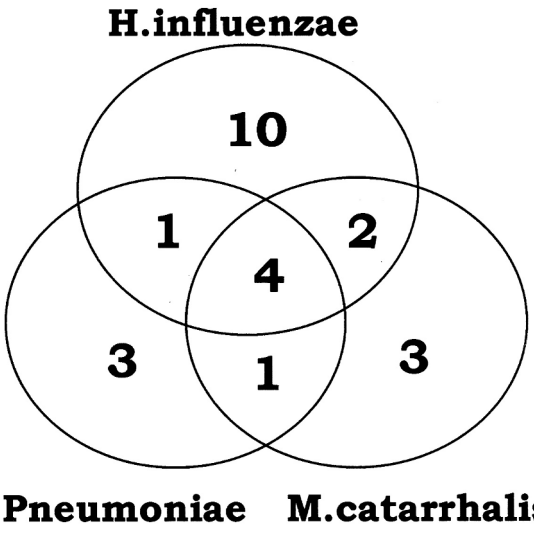

FIG. 2

Pathogens in middle-ear effusion (MEE) samples. About 70 per cent of detected *Haemophilus influenzae* was the sole infective organism, whereas *Streptococcus pneumoniae* and/or *Moraxella catarrhalis* were detected in conjunction with other pathogens.

samples from this group compared with MEE samples from patients who did not have such a history (7/10 versus 2/14; $p = 0.0187$).

Mutations in pbp genes of S. pneumoniae

PCR assays uncovered *pbp* gene mutations in eight of nine *S. pneumoniae* strains (Figure 1). All *S. pneumoniae* had mutations in at least two of the three *pbp* genes, i.e. two strains had mutations in *pbp2x* and *pbp2b* and six had mutations in *pbp1a*, *pbp2x* and *pbp2b*.

Discussion

A Eustachian tube dysfunction has classically been considered as the source of non-infective OME. However, recent molecular biological techniques as well as the present study have implicated bacteria in the aetiology of this condition.[2,5–7] While bacteria were detected in only four cultured MEE samples, PCR detected at least one of three pathogens in 32 per cent of MEE samples. The criticism against PCR with respect to detecting bacteria in MEE is the possibility that it might detect dead, and not live, bacteria. However, Rayner *et al.* demonstrated the presence of viable intact organisms which were metabolically active and yet not culturable by standard techniques, by showing the mRNA of *H. influenzae* in the 'culture-negative' MEE.[8] In addition, recent studies using animal models have uncovered direct evidence of pathogens attached to the middle-ear mucosa as a bacterial 'biofilm', rather than as free-floating organisms in MEE.[9] These findings further support the notion that PCR assay is superior to traditional culture methods when searching for bacteria in MEE samples.

Mills *et al.* studied the outcome of 225 children with OME. According to their study, in most of the children, a same type of effusion was found in both ears and an effusion type was same at initial and second surgery. These findings suggest that similar aetiological factors can produce the same effusion type.[10] Solzbacher *et al.* recently demonstrated that mucin in MEE inhibits the attachment of *H. influenzae* to mucosal epithelial cells.[11] Considering their reports, we hypothesized at the beginning of this study that OME with mucous and with serous MEE are different entities, as proposed by Sade.[12] That is, the mucosal glands of the middle ear might become mucin-rich to inhibit pathogens from attaching to the middle-ear mucosa and forming a mucous biofilm. On the other hand, OME with serous effusion might develop through a different mechanism such as a dysfunction of the Eustachian tube or adenoid vegetation, rather than via immune complexes or endotoxins related to infective pathogens in the middle ear. However, the present study found that the detection rates of pathogens in the mucous and serous types of MEE were quite similar, suggesting that pathogens are involved in the development of both types of MEE.[13]

- **This study investigates the microbial flora of middle-ear effusions in 52 children undergoing ventilation tube insertion**
- **Polymerase chain reaction was used to detect pathogens and to test the susceptibility of *Streptococcus pneumoniae* to penicillin**
- **High levels of *Streptococcus pneumoniae* were detected in the effusions of children with a long history of prior antibiotic administration, raising the possibility of a change in the bacterial flora due to antibiotic use**

Of interest, *S. pneumoniae* was significantly more frequently detected in refractory ears that required ventilation tube insertion at least twice. In addition, all isolated *S. pneumoniae* had mutations of the *pbp* genes. Since *S. pneumoniae* develops resistance via genomic alterations resulting in changes in cell membrane structures called penicillin-binding proteins (PBP),[14,15] these results show that antibiotic-resistant S. pneumoniae is associated with refractory OME.

Moreover, the detection rates of *S. pneumoniae* significantly differed between ears with and without prolonged exposure to antibiotics. Recent studies show that 38–43 per cent of middle-ear fluid is highly resistant to penicillin in the United States[16,17] and up to 60 per cent of healthy children harbour antibiotic-resistant *S. pneumoniae* in their nasopharynx.[18,19] Although the presence of antibiotic-resistant *S. pneumoniae* in MEE does not necessarily indicate serious infection, the present results demonstrate the increasing prevalence of antibiotic-resistant *S. pneumoniae* in the Japanese environment and the influence of prolonged exposure to antibiotics on its prevalence even in MEE.

https://doi.org/10.1258/002221505774783476 Published online by Cambridge University Press

References

1 Senturia BH. Studies concerned with tubotympanitis. *Ann Otol Rhinol Laryngol* 1958;**67**:440–67
2 Post JC, Preston RA, Aul JJ, Larkins-Pettigrew M, Rydquist-White J, Anderson KW, *et al.* Molecular analysis of bacterial pathogens in otitis media with effusion. *JAMA* 1995;**273**:1598–604
3 Hotomi M, Ubukata K, Konnno M, Samukawa T, Shimada J, Suzumoto M, *et al.* Identification of penicillin-resistant *Streptococcus pneumoniae* in nasopharynx of patients with acute otitis media by PCR. *Nippon Jibiinkoka Gakkai Kaiho* 1998;**101**:924–9
4 Shimada J, Hotomi M, Kuki K, Yamanaka N, Mitsuda T, Yokota S. Molecular analysis of pathogenes of upper respiratory tract infections in children - a study of nasopharyngeal *S. pneumoniae* and PBP genes in acute otitis media. *Nippon Jibiinkoka Gakkai Kaiho* 2000;**103**:552–9
5 Hotomi M, Tabata T, Kakiuchi H, Kunimoto M. Detection of *Haemophilus influenzae* in middle ear of otitis media with effusion by polymerase chain reaction. *Int J Pediatr Otorhinolaryngol* 1993;**27**:119–26
6 Hendolin PH, Markkanen A, Ylikoshki J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial specimens in middle ear effusions. *J Clin Microbiol* 1997;**35**:2854–8
7 Gok U, Bulut Y, Keles E, Yalcin S, Doymaz MZ. Bacteriological and PCR analysis of clinical material aspirated from otitis media with effusions. *Int J Pediatr Otorhinolaryngol* 2001;**60**:49–54
8 Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA* 1998;**279**:296–9
9 Post JC. Direct evidence of bacterial films in otitis media. *Laryngoscope* 2001;**111**:2083–94
10 Mills RP, Tay HL. Findings at initial surgery for childhood otitis media with effusion and subsequent outcome. *Clin Otol* 1995;**20**:461–4
11 Solzbacher D, Hanisch FG, van Alphen L, Gilsdorf JR, Schroten H. Mucin in middle ear effusions inhibits attachement of *Haemophilus influenzae* to mucosal epithelial cells. *Eur Arch Otorhinolaryngol* 2003;**260**:141–7
12 Sade J, Russo E, Fuchs C, Cohen D. Is secretory otitis media a single diseases entity? *Ann Otol Rhinol Laryngol* 2003;**112**:342–7
13 Giebink GS, Mills EL, Huff JS, Edelman CK, Weber ML, Juhn SK, *et al.* The microbiology of serous and mucoid otitis media. *Pediatrics* 1979;**63**:915–19
14 Dowson D, Hutchison A, Brannigan JA, George RC, Hansman D, Linares J, *et al.* Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae. Proc Natl Acad Sci USA* 1989;**86**:8842–6
15 Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, *et al.* Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. *J Antimicrob Chemother* 2001;**48**:915–18
16 Clary RA, Bahadori RS, Muntz HR, Lusk RP. Bacteria in the middle ear and nasopharynx during tympanostomy tube insertion. *Am J Otolaryngol* 1998;**19**:301–4
17 Sutton DV, Derkay CS, Darrow DH, Strasnick B. Resistant bacteria in middle ear fluid at the time of tympanotomy tube surgery. *Ann Otol Rhinol Laryngol* 2000;**109**:24–9
18 Masuda K, Masuda R, Nishi J, Tokuda K, Yoshinaga M, Miyata K. Incidences of nasopharyngeal colonization of respiratory bacterial pathogens in Japanese children attending day-care centers. *Pediatr Int* 2002;**44**:376–80
19 Petrosillo N, Pantosti A, Bordi E, Spano A, Del Grosso M, Tallarida B, *et al.* Prevalence, determinants, and molecular epidemiology of *Streptococcus pneumoniae* isolates colonizing the nasopharynx of healthy children in Rome. *Eur J Clin Microbiol Infect Dis* 2002;**21**:181–8

Address for correspondence:
Professor Ken-ichi Nibu,
Department of Otolaryngology-Head and Neck Surgery,
Kobe University, Graduate School of Medicine,
Kusunoki-cho 7–5–1, Chuo-ku,
Kobe 650–0017, Japan.

Fax: +81-78-382-6039
E-mail: nibu@med.kobe-u.ac.jp

Professor K-I Nibu takes responsibility for the integrity of the content of the paper.
Competing interests: None declared