Genetic Relatedness between Pneumococcal Populations Originating from the Nasopharynx, Adenoid, and Tympanic Cavity of Children with Otitis Media

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Previous studies have shown that *Streptococcus pneumoniae* exists in both middle ear effusions and the upper respiratory region from children with otitis media with effusion (OME), but it remains unclear whether these strains represent genetically identical clones. Therefore, it cannot be determined whether these bacteria originate from a common source. To determine the presence of pneumococci at different anatomical locations of OME patients, conventional culture and PCR techniques were used. To analyze the possible genetic relatedness between pneumococci from different anatomical sites, molecular typing by amplified fragment length polymorphism was utilized. The percentage of middle ear effusions of OME patients that are positive for pneumococci after PCR analysis (13%) was higher than after conventional culture (5%). Molecular fingerprints from pneumococci derived from two different anatomic sites within patients were very similar in 80% of OME patients and in 90% of acute otitis medium patients, indicating their genetic relatedness. Biofilm formation or pneumococcal L-forms probably play a role in OME, since culture-negative effusions prove to contain pneumococcal DNA. Bacteria involved in this process most likely originate from the nasopharynx since they show a close genetic relatedness with their nasopharyngeal counterparts.

Streptococcus pneumoniae, a major gram-positive human pathogen, is the most common cause of meningitis, sepsis, and middle ear infections in children and of pneumonia in immunocompromised and elderly individuals. Since antibiotic resistance has become a worldwide problem, which limits the choice of antimicrobial agents, prevention of pneumococcal diseases has become of great interest. However, despite major advances in the development of pneumococcal polysaccharide and pneumococcal conjugate vaccines leading to a reduction of invasive disorders (30), eradication of pneumococcal diseases is not within easy reach. More and more it seems likely that current vaccines will lack efficacy in the long term due to the genomic plasticity of the pneumococcus (e.g., capsular serotype transformation; interspecies transformation by uptake of pneumococcal DNA, as well as DNA of related species in the same ecological niche) (3, 29) and to serotype replacement (1). These challenges have led to further research into the mechanisms of the pathogenesis of pneumococcal disease in order to develop new therapeutic and preventive strategies (12).

Regarding the role of pneumococci in the pathogenesis of otitis media, the findings have been rather ambiguous. Although pneumococci usually dominate in the bacterial mix present in middle ear effusions during acute otitis media (AOM) (28), until recently the majority of middle ear effusions

in otitis media with effusion (OME) appeared to be culture negative (8, 11, 23, 24). The predominance of negative culture results has led to the hypothesis that OME results from an inflammatory response induced by residual bacterial metabolites subsequent to AOM (26). However, the introduction of new molecular techniques with significantly improved detection rates did show that in effusions that were negative by culture, bacterial DNA was present originating from often more than one bacterial species (e.g., *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*) (19, 20).

In addition to the identification of these pathogenic strains in middle ear effusions of children with otitis media *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* could also be cultured from the oropharynx (27), adenoid (13), and nasopharynx (9). Apparently, the upper respiratory region is an ideal habitat for these bacteria. Moreover, it has been demonstrated that samples simultaneously obtained from middle ear and nasopharynx from one patient sometimes contained identical bacterial strains or serotypes. From this it was concluded that microorganisms from the nasopharynx had entered the tympanic cavity via the Eustachian tube. However, this conclusion seems somewhat preliminary since it was not tested whether two different or two identical bacterial clones were involved, whereas isolates with the same serotype (and the same antibiotic resistance pattern) may yield different genotypic patterns (6). The value of this conclusion will certainly enhance if a genetic relatedness between the bacterial populations from both locations can be determined.

Therefore, the aim of the present study was to investigate in

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TABLE 1. Number of culture-positive samples/OME patient, distributed over respective locations, represented in percentages of the total amount of children with positive pneumococcal cultures

No. of culture-positive samples/patient	Location	Percentage of children:	
		From total study group	With genetically related samples ^a
1	Oropharynx or nasopharynx	36	
2	Oropharynx + nasopharynx (one patient: nasopharynx + middle ear fluid)	59	80 (2)
3	Oropharynx + nasopharynx + middle ear fluid	5	60 (3)

^a Numbers in parentheses indicate the number of sampled locations per patient.

a group of children with OME whether there is a genetic relatedness between pneumococci originating from middle ear, adenoid, and/or oropharynx. As a reference, a group of children with AOM was also selected for the present study.

MATERIALS AND METHODS

Patients. (i) OME. A total of 178 children (2 to 8 years old) were recruited from a population enrolled in a larger, randomized trial in which six hospitals participated. The selected children were the complete study groups of two participating hospitals in the city of Nijmegen, The Netherlands. In the trial, the efficacies of two therapies for recurrent OME were compared. Half of the children were treated with ventilation tubes only, while the other half received a 7-valent pneumococcal conjugate vaccine 21 to 28 days prior to insertion of the tubes (Wyeth Lederle Vaccines, Pearl River, NY).

(ii) AOM. In addition, 15 AOM patients (1 to 7 years old) were randomly recruited from a group of children with positive cultures from middle ear effusions and nasopharynx. These children belonged to a control group enrolled in a larger, randomized double-blind study to determine whether pneumococcal vaccination prevents recurrence of AOM in children with previous episodes of AOM (29). All children had experienced at least two episodes of AOM during the year before recruitment. Half of the group already had ventilation tubes. The control group received hepatitis A (Havrix Junior; GlaxoSmithKline, Zeist, The Netherlands) or hepatitis B (Engerix-B; GlaxoSmithKline) vaccinations.

For both the AOM and the OME studies, written parental informed consent was obtained before inclusion in the study. Both study protocols were approved by the appropriate medical ethics committees.

Collection and analysis of samples. In the OME study, samples from middle ear fluid (aspirated if present), oropharynx (swab), and adenoid biopsy were obtained during anesthesia for insertion of ventilation tubes. All samples were plated within 6 h onto two 5% Columbia blood agar plates, a 5% Columbia blood agar plate with 5 mg of gentamicin/liter, and a chocolate agar plate. Agar plates were incubated at 37°C for 48 h: the blood agar plates aerobically and anaerobically, the blood agar plate with gentamicin, and the chocolate agar plate with raised CO₂ (5%). Identification of bacterial strains was based on colony morphology and conventional methods of determination. When *S. pneumoniae* was isolated, a single colony was picked up for further analysis by immunological serotyping (Quellung reaction with commercially available antisera [Statens Seruminstitut, Copenhagen, Denmark]). Whenever pneumococci were simultaneously recovered from two or three locations (oropharynx/adenoid/middle ear effusion), molecular typing was performed and used for genotypic comparison.

In the AOM study, bacterial culture from middle ear fluid was obtained at the time of the first AOM episode occurring at least 1 month after the last vaccination. After clinical confirmation of the diagnosis of AOM, middle ear fluid was collected by myringotomy or by spontaneous drainage near the perforation site with an aspirator or a sterile dry cotton-wool swab. In addition, nasopharyngeal samples were obtained with a flexible, sterile, dry cotton-wool swab. Samples were processed as described above. For the present study, 15 children with positive pneumococcal cultures both from middle ear effusion and from nasopharynx were selected and molecular typing was performed.

Detection of pneumococcal DNA by *lytA* PCR. DNA was isolated from 90 µl of middle ear effusion by using a MagNA Pure automated DNA extraction platform (Roche Diagnostics, Almere, The Netherlands) as recommended by the manufacturer. Detection of the autolysin gene was performed by a real-time PCR assay targeting the *lytA* gene with primers and probes described by McAvin et al. (15).

Appropriate positive and negative controls were used. To check for the pres-

ence of PCR inhibitors that may compromise the amplification reaction, an exogenous DNA target was added to the specimens prior to DNA extraction. This artificial DNA construct also served to verify proper functioning of all reagents and equipment. This target was amplified in a separate PCR.

Molecular typing by AFLP. DNA was isolated from colonies by using a MagNA Pure automated DNA extraction platform. Prior to extraction, cells were incubated with 50 U of mutanolysin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 15 min at 37°C. Amplified fragment length polymorphism (AFLP) analyses were performed according to established protocols (14, 16) with the restriction enzyme combination EcoRI and MseI (Westburg, Leusden, The Netherlands). For amplification, a fluorescein-labeled EcoRI primer without extensions and an MseI primer extended with a G residue were used. Obtained amplification products were analyzed on a MegaBACE automated DNA analysis platform (Amersham Biosciences, Roosendaal, The Netherlands) under conditions recommended by the manufacturer.

Fingerprint patterns were analyzed for similarity using Gel Compar II software (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed by using the unweighted pair-group method with arithmetic averages with the Pearson correlation coefficient, expressed as a percentage of similarity. Strains were considered to be clonally related if proven to be >90% identical.

RESULTS

Bacterial culture. Samples for pneumococcal culture were collected from 178 children with OME. In the samples of 110 children with OME (62%), growth of pneumococci in the standard culture assay was detected, distributed over 1, 2, or 3 samples per patients. From only a minority (5%) of children with OME *S. pneumoniae* could be cultured from middle ear effusions. The results are summarized in Table 1.

PCR. Middle ear effusions were tested by *lytA* PCR for the presence of pneumococcal DNA. Since fluid could not be collected from all patients at the moment of insertion of ventilation tubes, not all ears were tested. A total of 165 middle ear effusions were analyzed. The results of PCR analysis and corresponding pneumococcal culture have been summarized in a table (Table 2).

In all, pneumococcal DNA could be detected in 21 samples (13%). All AOM samples were PCR positive, as was expected because they were culture positive. Positive and negative controls yielded positive and negative PCR results, respectively.

TABLE 2. Presence of pneumococci in middle ear fluids as detected by standard culture and PCR, represented in percentages of the total number of effusions that were analyzed

Percentage of effusions	Standard culture	PCR
10	Negative	Positive
3	Positive	Positive
87	Negative	Negative

FIG. 1. Genetic relatedness between pneumococcal isolates from different locations within one patient and between patients based on AFLP fingerprints. The scale bar indicates the percentage of similarity. (A) Random selection of samples from OME patients; (B) samples from AOM patients. Serotypes per sample are included. NT, not typeable. Isolates are labeled as follows: NAS, isolates obtained from nasopharyngeal specimens (adenoid biopsies); ORO, oropharyngeal specimens (throat swab); and MEF, middle ear fluid specimens.

The results also indicated that there was no evidence for PCR inhibitors in the samples (results not shown).

AFLP. AFLP results are depicted in dendrograms in Fig. 1. (This figure also includes the results of serotyping.) One molecular pneumococcal fingerprint was obtained per anatomic site. These were compared to assess their genetic relatedness.

Dendrogram B is based on a comparison of fingerprints from all pneumococcal isolates belonging to the AOM study

group. Dendrogram A is based on a comparison of fingerprints from a representative, random selection of pneumococcal isolates belonging to the OME group (the dendrogram based on all isolates of the OME group is too large to be reproduced on one page). The percentages discussed below, however, are derived from the complete dendrogram.

In 60% of the children with OME from which pneumococcal isolates were available derived from three locations within one

patient (adenoid, oropharynx, and middle ear fluid), the degree of genetic relatedness between pneumococcal isolates per patient indicated a clonal origin (Table 1). In the group of OME children from which pneumococcal samples were derived from two locations (adenoid and oropharynx), clonal relatedness between paired pneumococcal isolates per patient was observed in 80% of the children (Table 1).

In the group of AOM children, almost 90% of the paired pneumococcal fingerprints per patient (derived from nasopharynx and middle ear effusion) showed a clonal origin.

DISCUSSION

Dendrograms resulting from AFLP fingerprint analysis demonstrate that pneumococcal genotypes per patient show a strong resemblance to one another in 80% of all OME patients when paired samples, as well as three samples per patient, are compared. Furthermore, 90% of the paired pneumococcal samples from AOM patients show similarity. A comparison of serotyping and genotyping of paired pneumococcal samples of AOM patients demonstrates that identical serotypes correspond to similar genetic fingerprints. However, identical serotypes of paired pneumococcal samples derived from OME children do not always display homogeneous fingerprint patterns. Identical serotypes demonstrating heterogeneous genotypes are frequently observed in pneumococci since these bacteria have the genetic capacity to switch serotype. Moreover, transfer of genes between strains is possible (10). Since OME is a chronic disease, the period between onset and sampling is much longer than in AOM; hence, it is plausible that serotype switch and/or gene transfer becomes evident especially in OME. Genotypic similarity, as was found in the majority of the paired samples, indicates clonal relatedness and implicates that pneumococci present at different sites in the upper respiratory region of a patient originate from a single source. This pneumococcal source is most likely located in nasopharynx or throat, since these zones are open to the external environment. These sites are easily colonized by bacteria and include the orifices of the Eustachian tubes that are localized at both sides in the nasopharynx. It is conceivable that in spite of the protective mechanisms that are present in the Eustachian tube, bacteria may be able to migrate from the nasopharynx into the middle ear cavity under specific conditions (e.g., pressure differences between the nasopharynx and the middle ear cavity or an immature or compromised immune system), subsequently leading to otitis media. This assumption is strengthened especially by the observation of clonal relatedness between pneumococci present in the nasopharynx and middle ear in 90% of AOM patients.

In pneumococcal samples obtained from a minority of patients, different fingerprint patterns were found from different locations within one patient, which indicates that these pneumococci are not genetically related. An explanation for this can be found in the method. Although most children are colonized by several pneumococcal serotypes (1, 29), only a single colony from each culture was selected for further analysis because it was considered highly likely that this one colony will represent the most prevalent serotype in the pneumococcal population at that location. Obviously, the more colonies tested, the better the diversity of the pneumococcal population can be explored,

but to achieve statistical significance this approach would be extremely time-consuming and expensive. Considering that the most uncommon serotype represents only 5% of the total pneumococcal population, at least 59 colonies from each sample would have to be serotyped to obtain 95% probability of collecting the second pneumococcal type (2, 7).

The differences between AOM and OME patients are remarkable. A total of 21% of AOM effusions were culture positive for pneumococci (29), whereas in OME this percentage was only 5%. This finding may indicate AOM to be an active inflammatory process and OME to be a chronic disease. Comparison of fingerprints from nasopharyngeal pneumococci and middle ear pneumococci from one patient showed clonal relatedness in 90% of AOM patients. However, when pneumococcal fingerprints of OME children were compared (isolated from isolates obtained from nasopharyngeal specimens [adenoid biopsies], oropharyngeal specimens [throat swab], and middle ear fluid specimens), a clonal relationship was observed in 60% of the children. This may indicate that in AOM patients pneumococcal translocation via the Eustachian tube has occurred recently, whereas in OME patients pneumococcal passage may have occurred earlier. During that interim period the nasopharyngeal pneumococcal population may have changed, which may affect the degree of clonal relatedness.

Both conventional bacterial culture and *lytA* PCR were applied in the present study to establish the presence of pneumococci in middle ear fluids of children with OME. PCR analysis revealed a higher percentage of positive middle ear effusions (13%) than expected by pneumococcal culture (5%), which is in line with the results of Pereira et al. (17). However, Post et al. (19) evaluated a higher percentage of pneumococcus-positive effusions by using PCR (29.9%), although the percentage determined by conventional culture was similar (5%). This discrepancy may be due to a difference in PCR target. Post et al. applied a PCR method that targeted penicillin-binding protein gene 2B (*PBP2B*) (19). Given the fact that horizontal transfer of *PBP* genes may occur from pneumococci to viridans group streptococci present in the same niche (18), a PCR targeting the *PBP2B* gene may lead to the amplification of putative pneumococcal sequences, resulting in false positivity, whereas the *lytA* PCR method is of proven specificity (16).

Observations of negative or minor bacterial growth by culture, despite considerable amounts of bacterial DNA in effusions of OME patients, may be indicative of the presence of biofilms. Recently, it has been shown that bacterial biofilms were formed on the middle ear mucosae of chinchillas subsequent to experimental induction of otitis media by *Haemophilus influenzae* (5, 21). In addition, biofilms were found to be present on tympanostomy tubes derived from OME patients (21). These observations strongly indicate that biofilm formation may play a role in the pathogenesis of OME. In that case it is quite likely that only bacteria released from the biofilm are detected by culture.

Minor bacterial growth in middle ear effusions of OME patients may also be explained by the concept described by Domingue et al. (4), illustrating that bacteria exposed to a deleterious environment (i.e., host defense) can change into L forms that persist in a latent state within the host, causing pathological responses. These bacteria will not be detected by

conventional culture methods because L forms do not form colonies under standard culture conditions. PCR may offer an alternative for culture analysis, although a drawback of this technique is that the nature of the identified DNA remains uncertain, since DNA of both live and dead bacteria may be amplified by PCR. However, from a study of Post et al. (22) it can be concluded that DNA in effusions originates from live bacteria, since these authors demonstrated that DNA from nonviable bacteria lacks persistence in middle ear effusions of chinchillas. In addition, bacterial mRNA was identified in culture-negative middle ear effusions of children with OME (25), which indicates the presence of viable, metabolically active, intact organisms, since bacterial mRNA has a very short half-life (several seconds to minutes). To establish whether bacterial L forms or biofilm bacteria are involved in OME, mucosal biopsies from several locations in the tympanic cavity are required, which for medical ethical reasons cannot be obtained from patients with OME only.

Although the exact bacterial *modus vivendi* remains unclear, we have shown here that pneumococci in middle ear effusions of OME and AOM patients most likely originate from the nasopharynx, since they show close genetic relatedness to their nasopharyngeal counterparts.

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