Correlation between Presence of Viable Bacteria and Presence of Endotoxin in Middle-Ear Effusions

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The presence of endotoxin (detected by the Limulus amebocyte lysate assay) was compared to the presence of viable Haemophilus influenzae and Moraxella catarrhalis (detected by PCR) in 106 middle-ear effusions from pediatric patients with chronic otitis media. Endotoxin was found in 81 of the 106 specimens. Of these 81 specimens, 66 (81.5%) also tested positive for one or both of the gram-negative bacteria H. influenzae and M. catarrhalis. The data suggest that viable gram-negative bacteria, detectable by PCR but often undetectable by culture, may be the source of endotoxin in middle-ear effusions.

Otitis media is the most common reason for an ill child to visit a health-care provider and receive antimicrobial treatments or surgery (16, 17). Chronic otitis media with effusion (OME), the persistence of fluid in the middle ear with minimal constitutional symptoms, can lead to significant hearing loss and delayed speech development in pediatric patients (2, 8). Common reasons for the onset of OME include dysfunction of the eustachian tube, immaturity of the immune system, allergic response, and infection.

Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis are the most common bacteria cultured from middle-ear-effusion aspirates; however, the majority of effusions (40 to 60%) are negative by culture for any bacterium (1, 5, 7). Recent application of PCR has allowed for the simultaneous and specific detection of DNA from S. pneumoniae, H. influenzae, and M. catarrhalis in culture-sterile specimens from pediatric patients with chronic OME (13). Additional studies demonstrated that in the chinchilla middle ear, purified DNA and DNA from pasteurized bacteria are rapidly degraded, as demonstrated by the fact that they are not detectable by PCR (14). Conversely, inoculated live bacteria persist for weeks, and their DNA is detectable by PCR during that time, though after treatment with antibiotics the effusions in which this DNA is detected are sterile as determined by culture. These experiments showed that any bacterial DNA detected by PCR in middle-ear effusions comes from viable bacteria. Furthermore, the bacteria within culture-sterile pediatric middle-ear effusions have been shown to be metabolically active by means of reverse transcriptase PCR-based assays (15). Viable, metabolically active H. influenzae, the most common bacterium isolated from the effusions of patients with OME, was present in approximately 30% of culture-sterile middle-ear effusions and 100% of culture-positive middle-ear effusions (15).

Endotoxin, a lipoooligosaccharide complex on the outer surface of most gram-negative bacteria, is thought to be largely responsible for the inflammatory response and the accumulation of fluid in the middle ear in OME patients (4). However, it has been unclear whether the effusion-inducing endotoxin can persist in the middle ear only as a component of viable bacteria or whether it can persist as a component of nonviable bacteria. In a previous study, endotoxin was present in 80% of all middle-ear effusions, including 67% of effusions that were negative as determined by culture for any bacterium (5). This result indicated that cultivable bacteria were not present in the majority of endotoxin-positive specimens. However, the culture method used to detect bacteria in that earlier study is less sensitive than PCR and would not have identified viable, nondividing bacteria associated with a biofilm. We previously demonstrated that PCR detection of bacteria correlates nearly 100% with the presence of metabolically active pathogens (15). Viable, nondividing gram-negative bacteria may harbor endotoxin, but because they are not detectable by culture they would have been overlooked as a source of lipoooligosaccharide.

In our study, we attempted to correlate the detection of endotoxin, using the Limulus amebocyte lysate (LAL) assay, with the presence of viable H. influenzae and M. catarrhalis, as detected by PCR. We hypothesize that the greater sensitivity of PCR over culture will more definitely establish the origins of bacterial endotoxin in chronic OME.

Patient population. A total of 106 middle-ear-effusion specimens were collected from pediatric outpatients at Children's Hospital of Pittsburgh during myringotomy and tube placement for chronic OME. Patients ranged in age from 3 months to 13 years.

An approved otoscopyist diagnosed all patients, and tympanometric evaluation was performed. The criteria for myringotomy and tube placement included the presence of middle-ear fluid for at least 3 months despite multiple courses of antimicrobial therapy, which included agents against β-lactamase-producing organisms. Treatment was not influenced by the results of this study.

Acquisition of clinical specimens. The external ear canal was disinfected and desquamated by placing 70% isopropyl alcohol in the ear canal for 1 min. Immediately following myringotomy, the effusion was suctioned from the middle-ear cleft with a sterile 14-gauge Baxter Quick-Cath cover attached to a Sentu-
ria trap. In order to clear the catheter of viscous material, prior to the procedure 50 μl of sterile saline was added to each autoclaved collection tube and used to dilute the effusion aspirate. Each sample was snap-frozen on dry ice and transported to the Center for Genomic Sciences (Pittsburgh, Pa.), where it was stored at ~80°C before molecular analyses.

Preparation of clinical specimens for PCR. The sensitivity and specificity of the PCR assay for *H. influenzae* and *M. catarrhalis* were established as previously described (13). After the effusions were thawed, 50 μl was aliquoted for the endotoxin assay, and the remainder was used for the DNA extraction. The Trizol LS reagent (Life Technologies, Gaithersburg, Md.) protocol for RNA and DNA extraction was used according to the manufacturer’s recommendations, with one modification. The nucleic acid specimens used for multiplex PCR were not treated with DNase.

PCR. Multiplex PCR and amplification analyses were performed for *H. influenzae* with primer set HI-IV/V/VIB and for *M. catarrhalis* with primer set MCAT51/52/53 as described previously (13).

LAL endotoxin assay. The presence of endotoxin was determined with the BioWhittaker (Walkersville, Md.) QCL-1000 third-generation chromogenic LAL assay. The assay was performed as previously described, with several modifications (11). Endotoxin concentrations were determined with a Perkin-Elmer spectrophotometer; the absorbance of each specimen was measured at 405 nm and was used to calculate the endotoxin concentration. Escherichia coli standardization dilutions ranged from 0.1 to 1.0 endotoxin units (EU) per ml (9 EU = 1 ng). A calculated value of 0.1 EU/ml was considered the threshold for endotoxin positivity in the specimens. Arithmetically positive values below this threshold were recorded, but their significance is unknown. Arithmetically negative values calculated for endotoxin were assumed to be 0.0 EU/ml. The six below-threshold specimens that tested positive by PCR for both *H. influenzae* and *M. catarrhalis* had the highest of these low values, but even these reached an average endotoxin concentration of only 0.0397 EU/ml.

Table 2 shows the correlation. Overall, 81 of the 106 middle-ear-effusion specimens (76.4%) tested positive for endotoxin. Twenty-five (23.6%) specimens were below the LAL assay threshold for endotoxin. Of the endotoxin-positive specimens, 66 (81.5%) were positive by PCR for *H. influenzae* and/or *M. catarrhalis*.

Discussion. The percentage of endotoxin-positive middle-ear-effusion specimens (76.4%) is comparable to the result (80%) reported by DeMaria et al. (5, 18). The PCR technique for the detection of *H. influenzae* and *M. catarrhalis*, however, identified bacteria in 81.5% of endotoxin-positive effusions, while in the previous study (5) culture had detected bacteria in only 33% of the effusions. The greater sensitivity of PCR (247%) over culture in these studies closely reflects the results seen by Post et al. (268%) (12) and Rayner et al. (264%) (15), who directly compared PCR and culture data for all middle-ear effusions.

Bacterial endotoxin has been attributed to the release of inflammatory mediators by macrophages (10). Earlier work showed that residual endotoxin from pasteurized bacteria could induce an effusion in the bullae of the chinchilla (4). Additionally, it has been shown that the majority of endotoxin-positive effusions from patients with OME (67%) are sterile as determined by culture for any bacteria (5). These studies led to the postulate that residual endotoxin might cause OME to persist in the absence of viable bacteria (5). The PCR-based assays for the detection of bacteria used in this study show that viable bacteria exist in a larger percentage of endotoxin-positive effusions (81.5%) than had been seen with the culture method of detection (33%). Thus, while the presence of endotoxin is most likely important for the persistence of OME (5), our results suggest that viable, nonculturable gram-negative bacteria produce the endotoxin.

The correlation of average endotoxin concentration and PCR detection of bacteria indicates that there is an increase in endotoxin concentration when more than one species of bacteria exist in an effusion. In the samples where only one of the two bacteria tested for by PCR was detected, the average concentration of endotoxin was approximately 0.9 EU/ml.

### Table 1. Endotoxin concentrations in specimens of pediatric middle-ear effusions

<table>
<thead>
<tr>
<th>Bacterium detected by PCR</th>
<th>Endotoxin positive No. of specimens</th>
<th>Avg concn (EU/ml)</th>
<th>Below LAL assay threshold (no. of specimens)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em> only</td>
<td>36</td>
<td>0.9435</td>
<td>10</td>
</tr>
<tr>
<td><em>M. catarrhalis</em> only</td>
<td>4</td>
<td>0.9205</td>
<td>0</td>
</tr>
<tr>
<td><em>H. influenzae</em> and <em>M. catarrhalis</em></td>
<td>26</td>
<td>1.6338</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>15</td>
<td>0.5798</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table 2. Correlation of endotoxin concentration (determined by LAL assay) and presence of bacteria (determined by PCR) in the 106 specimens**

<table>
<thead>
<tr>
<th>Result of test for:</th>
<th>Endotoxin</th>
<th>No. (%) of specimens</th>
<th>Avg endotoxin concn (EU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>66 (62.3)</td>
<td>1.214</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>15 (14.2)</td>
<td>0.5798</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>16 (15.1)</td>
<td>0.3608</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>9 (8.5)</td>
<td>0.0397</td>
</tr>
</tbody>
</table>

*Endotoxin was below the threshold of the LAL assay.*
When both bacteria were detected, endotoxin increased approximately 75% to 1.6 EU/ml. However, the endotoxin concentrations of many of the samples that tested positive for both bacteria had absorbance readings that exceeded the linear range of the spectrophotometer. Therefore, the measured increase in average concentration is probably an underrepresentation of the actual increase. Further studies will be needed to determine the exact endotoxin concentrations of PCR-positive specimens.

The correlation between bacterial detection by PCR and endotoxin detection by LAL assay was 70.8% (75 of 106 specimens). There were 31 discordant specimens, 16 of which were negative for endotoxin (below the LAL assay detection threshold) and positive for bacteria (as determined by PCR). These discordant specimens might indicate a limitation of the LAL assay. Levin et al. demonstrated that some proteins can act as inhibitors of the LAL assay, thereby reducing the endotoxin concentration measurement (9). The remaining 15 discordant specimens were positive for endotoxin and negative for bacteria by PCR. These samples might be indicative of endotoxin embedded within nonviable bacteria, residual endotoxin, nonspecificity of the LAL assay (6), or, more likely, the existence of some additional gram-negative bacteria not tested for by PCR in this study.

The PCR and endotoxin data in this study support the hypothesis that chronic OME is a biofilm disease. A biofilm is a community of bacteria that have adhered to a surface or to each other (3). The attachment of bacteria to a surface triggers the expression of a cassette of genes, which results in the formation of a biofilm. The “biofilm phenotype” confers reduced metabolic activity and enhanced antibiotic resistance in comparison with the planktonic phenotype. The existence of a biofilm in the middle ear in patients with chronic OME would explain why bacteria are detectable by PCR and not by culture (15), since biofilm bacteria have been demonstrated to be highly resistant to growth in standard planktonic culture, probably because of differences in gene expression (3). It can also be postulated that, following the death of bacteria within a biofilm, detection by PCR would cease and endotoxin would persist embedded within the biofilm matrix until that matrix degraded.

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REFERENCES