Comparison of direct-plating and broth-enrichment culture methods for detection of potential bacterial pathogens in respiratory secretions

Ravinder Kaur, Jareth Wischmeyer, Matthew Morris and Michael E. Pichichero

Abstract

Objective. We compared the recovery of potential respiratory bacterial pathogens and normal flora from nasopharyngeal specimens collected from children during health and at the onset of acute otitis media (AOM) by selective direct-plating and overnight broth-enrichment.

Methods. Overall, 3442 nasal wash (NW) samples collected from young children were analysed from a 10-year prospective study. NWs were cultured by (1) direct-plating to TSAII/5% sheep blood agar and chocolate agar plates and (2) overnight broth-enrichment in BacT/ALERT SA-broth followed by plating. Standard microbiology techniques were applied to identify three dominant respiratory bacterial pathogens: *Streptococcus pneumoniae* (Spn), *Haemophilus influenzae* (Hflu) and *Moraxella catarrhalis* (Mcat) as well as two common nasal flora, *Staphylococcus aureus* (SA) and alpha-haemolytic *Streptococci* (AHS).

Results/Key findings. Direct-plating of NW resulted in isolation of Spn from 37.8%, Hflu from 13.6% and Mcat from 33.2% of samples. In comparison, overnight broth-enrichment isolated fewer Spn (30.1%), Hflu (6.2%) and Mcat (16.2%) (P<0.001–0.0001). Broth-enrichment resulted in significant increased isolation of SA (6.0%) and AHS (30.1%) (P<0.0001). Competition between bacterial species in broth when both species were detected by direct-plating was assessed, and it was found that SA and AHS out-competed other species during broth-enrichment when samples were collected from healthy children but not during AOM. In middle ear fluids (MEF) at the onset of AOM, broth-enrichment resulted in higher recovery of Spn (+10.4%, P<0.001), Hflu (+4.4%, P=0.39) and Mcat (+13.5%, <0.001).

Conclusion. Broth-enrichment significantly reduces the accurate detection of bacterial respiratory pathogens and increases identification of SA and AHS in NW. Broth-enrichment improves detection of bacterial respiratory pathogens in MEF samples.

INTRODUCTION

Colonization of the nasopharynx (NP) by potential respiratory bacterial pathogens is frequent in early childhood and is the first step in pathogenesis of invasive diseases and respiratory tract infections such as pneumonia, sinusitis and acute otitis media (AOM) [1–4]. The predominant respiratory bacterial pathogens that colonize the NP of children are *Streptococcus pneumoniae* (Spn), non-typeable *Haemophilus influenzae* (Hflu) and *Moraxella catarrhalis* (Mcat) [1]. Other normal nasopharyngeal bacterial flora, *Staphylococcus aureus* (SA), and alpha-haemolytic *Streptococci* (AHS) are also common [2, 5]. Interactions and competition between these species give rise to the complex dynamics of the NP microbiome [2]. Understanding the interactions between bacterial species and the regulation of density, virulence factor expression and the capacity to produce disease will likely provide new approaches for disease prevention and treatment. Thus, surveys of species present in the NP during different stages of pathogenesis are of importance.

When bacterial recovery of potential respiratory bacterial pathogens is the ultimate goal of sample analysis, selective *in vitro* enrichment can be beneficial. As part of an ongoing prospective study of NP colonization by potential respiratory bacterial pathogens in children 6–30 months old, we have reported the recovery of the three main potential respiratory bacterial pathogens (Spn, Hflu, Mcat) from nasal wash (NW) samples [6–12]. In an attempt to obtain...
maximal recovery of potential respiratory bacterial pathogens, we performed direct culture by streak-plating the NW onto blood and chocolate agar plates. At the same time, we performed overnight enrichment of samples in a BacT/ALERT SA-broth-enrichment medium (BD Biosciences). Broth was incubated for 16–20 h, followed by streak-plating. In this study, we sought to understand the benefits and disadvantages of broth-enrichment for the purpose of detecting potential respiratory bacterial pathogens in the NP.

METHODS

Study populations

Details of the study design have been previously described [8, 9]. Children were enrolled into the study at the age of 6 months and prospectively followed until 30–36 months of age during 2006–2016. Children were healthy, full term birth, no craniofacial anomalies and no known immune deficits. All children had received all doses of pneumococcal conjugate vaccine (PCV) according to the U.S. schedule; either PCV7 or PCV13 depending on the date of their enrollment, along with other routine childhood vaccinations. Written informed parental consent was obtained prior to study procedures as approved by the University of Rochester IRB and subsequently by the Rochester General Hospital IRB.

NP sample collections and processing

NP wash samples were collected from children by instillation and aspiration of 1 ml of sterile PBS into each nare and collecting the solution in a sterile container. On arrival to the laboratory, 2–3 h later, 50–100 µl of the sample was transferred onto BD Tryptic Soy with 5% sheep blood agar and BD Tryptic Soy chocolate agar plates and dilution streaked to promote single colony growth. Plates were incubated overnight at 37 °C, 5% CO₂. An additional 100 µl of NW were transferred into 2 ml of BacT/Alert SA culture media broth and incubated overnight at 37 °C, 5% CO₂. After incubation, the broth was plated onto chocolate and blood agar and subsequently dilution-streaked and incubated to allow for bacterial growth. BacT/Alert SA media has been shown to recover/grow all potential respiratory pathogens of interest [13].

Middle ear fluid (MEF) collection and processing

MEF samples, collected by tympanocentesis, were processed in the same manner as NW samples when the children developed AOM to evaluate the impact of direct culture versus the broth-enrichment of MEF [7, 8].

Isolation and identification of micro-organisms from specimens

Bacterial isolates were identified from the NW and MEF samples by standard clinical microbiology procedures [14–16]. Briefly, Spn was identified using colony morphology, α-haemolysis, P-disc sensitivity test. Identification of Spn from AHS was conducted by the implementation of the P-disc. Serotypes of Spn were determined by latex agglutination (Pneumotest-Latex, Statens Serum Institute, Copenhagen, Denmark) according to the manufacturer’s instructions. Quellung reactions were used to identify the serotype subgroup. An isolate was identified as Hflu based on colony morphology, growth requirement for hemin and nicotinamideadenine dinucleotide using Haemophilus ID QA plates. For Mcat testing grey-white hemispheric colonies of waxy surface were identified using hockey puck test and catarrhalis disc test. All Hflu and Mcat isolates were tested for β-lactamase production with the chromogenic cephalosporin disk method. SA isolates were identified based on colony morphology, clear β-haemolysis on blood agar plates, catalase positive test and coagulate positive test.

Data analysis

The NW direct-plating and NW broth-enrichment culture results were compared during health and at the onset of AOM illness; MEF and MEF broth-enrichment culture results were also compared. We used the chi-square test in GraphPad Prism version 6.1 for windows. *P*-values of <0.05 were considered significant. F plots of bacterial species interactions in broth were prepared using the ggplot2 package in R version 3.1.1 [17, 18].

RESULTS

A total of 2824 healthy and 890 AOM illness visit samples were included in the analysis obtained from 750 children between June 2006 and September 2016. Overall 78% of visits had no antibiotic usage up to 30 days before the visit date. Separating the visits from healthy and AOM visits, healthy children had not taken any antibiotics within the 30 days before the visit 82% of the time, and AOM children had not taken antibiotics 57% of the time. Different otopathogens’ recovery over time has been shown previously in different publications [6, 9, 10, 19].

NW direct-plating and broth analysis

First we compared total bacterial recovery from NW by direct-plating and after broth-enrichment from the 2824 healthy visit samples. Data was not quantitatively determined for each bacterial pathogen and results are only based on whether a particular pathogen was present or not. Almost all (>99% of cases) of the Mcat strains isolated were β-lactamase positive. Among Hflu, ~25% were β-lactamase positive. Details of antibiotic susceptibility has been shown previously [6, 9–12, 19]. Isolation rates of Spn, Hflu, Mcat, SA, AHS and uncharacterized bacteria (other) by direct-plating and after broth-enrichment are shown in Fig. 1. A significant reduction in the isolation frequency of potential respiratory bacterial pathogens (Spn, Hflu and Mcat) during both healthy (Fig. 1a) and AOM onset visits (Fig. 1b) was observed, suggesting that broth-enrichment does not enhance the recovery of potential respiratory bacterial pathogens. In contrast, recovery of SA, AHS and uncharacterized bacteria was significantly increased after broth-enrichment in both healthy and AOM illness visit NP.
samples, suggesting that these species grew more rapidly in broth than potential respiratory bacterial pathogens.

The recovery of bacteria from MEF collected during AOM was not significantly affected by broth-enrichment, apart from AHS, where broth-enrichment resulted in significantly less recovery 1.9% as compared to 4.7% by direct-plating (P<0.01) (Fig. 1c). The middle ear site is usually a non-culturable otopathogen-free site so no normal floras are present.

**NW bacterial recovery after broth-enrichment**

Table 1 shows the percentage of bacteria recovered after broth-enrichment when bacteria failed to grow by direct-
plating. In NP samples collected during health, Spn was recovered from broth-enriched samples but not by direct- plated samples in 4.3 % of cases, Hflu in 7.3 % of cases and Mcat in 3.4 % of cases. In NW samples at the onset of AOM, Spn, Hflu and Mcat were recovered from broth-enriched but not direct-plated samples in 3.5, 2.8 and 5.0 % of cases, respectively. SA (54 % during health, 67 % during AOM) and AHS (32 % health, 67 % AOM) were significantly more likely to be isolated after broth-enrichment.

**MEF bacterial recovery after broth-enrichment**

In MEF samples broth-enrichment resulted in increased isolation of Spn (+10.4 %, P<0.001), Hflu (+4.4 %, P=0.39) and Mcat (+13.5 %, <0.001) (Table 1). SA (44.4 %) and AHS (15.9 %) were also more likely to be isolated after broth-enrichment. This data indicates that broth-enrichment has value to improve potential respiratory bacterial pathogens’ identification in MEF.

**In vitro bacterial–bacterial competition with the inocula present naturally in NP**

To assess *in vitro* competition between bacterial species during broth-enrichment, we calculated the likelihood of recovering only one species after overnight enrichment of samples where two species were isolated by direct-plating in healthy and AOM visits. Fig. 2 shows the results of how different bacterial species out-compete each other in broth samples when more than one species was present in NW. When Spn was isolated by direct-plating along with Hflu (294 cases), Mcat (706 cases) or AHS (72 cases), Spn alone was isolated after enrichment in at least 50 % of cases. When Spn was isolated by direct-plating along with SA (18 cases), Spn was isolated alone after enrichment in less than 50 % of cases. Spn was more likely to out-compete AHS during enrichment of NP samples collected during AOM (P<0.05). Hflu and Mcat tended not to out-compete other species during broth-enrichment. Interestingly, the normal flora AHS usually out-competed other species in broth samples, but were more likely to do so in samples collected during health. AHS showed significantly lower competition with Hflu and Mcat (P<0.05) in AOM visits in comparison with healthy visits.

Comparisons between the out-competition rates of different species during healthy and AOM visits is also shown in Fig. 2. During the healthy state, AHS and SA were equally likely to out-compete Spn, and both species were more likely than Hflu or Mcat to out-compete Spn. At the onset of AOM only SA out-competed Spn. To further demonstrate the phenomena, we measured the likelihood of different species appearing in the broth-enrichment of samples where only one species was isolated by direct-plating of NW. AHS more consistently appeared (15–40 %) after the broth-enrichment of samples where only Spn, Hflu or Mcat was isolated by direct-plating. Since, in most of the MEF sample cases, only one of the potential otopathogens was recovered, no significant competition was observed (data not shown).

**DISCUSSION**

Broth-enrichment is frequently used to increase the detection of bacterial respiratory pathogens especially when the transfer of samples to the laboratory might be prolonged. In this report, we analysed 10 years of data to measure the benefits and disadvantages of direct-plating versus broth-enrichment and found that broth-enrichment marginally increased recovery of bacterial respiratory pathogens from the NP and at the expense of a marked increase in recovery by non-pathogenic normal flora. Broth-enrichment of MEF samples was shown to be of value. Competition between species during broth-enrichment resulted in normal flora out-competing bacterial respiratory pathogens with reproducible patterns.

Based on the most basic observation of comparing the number of species isolated by direct-plating versus broth-enrichment, a significant reduction in the number of bacterial respiratory pathogens isolated from NW occurred with enrichment during both healthy and AOM visits. This suggests that broth-enrichment is disadvantageous because it reduces recovery of the target isolates. However, if the goal is to isolate every possible bacterial respiratory pathogen present in the NP at the time of sampling, then an increase of 3.4–7.3 % at healthy visits and 2.8–5.0 % at AOM visits might be anticipated from broth-enrichment processing at the expense of more technical time to identify the target organisms among the abundant normal flora.

The increased number of bacterial respiratory pathogens recovered from MEF samples after broth-enrichment was significant and since the middle ear is typically non-culturable or home to only species of bacterial respiratory pathogen during AOM the enrichment might be endorsed.

Bacterial species are known to compete for nutritional resources and for dominance of the NP niche [20]. Spn and Hflu are frequently found to co-colonize the NP. It has been shown that Hflu out-competes Spn for survival through signaling of nucleotide-binding oligomerization domain-1 (Nod1) to facilitate host clearance of Spn [21], but when grown in broth, we found that Spn predominates over Hflu.

It has been shown that Spn and SA are usually recovered from different sites in the respiratory tract (posterior NP for Spn and anterior nares for SA) [22, 23]. Our low rates of SA recovery might be due to our sampling process: we perform NP washing by insertion of a bulb syringe securely into the nares to obtain a seal for adequate recovery of the saline

---

Table 1. Percentage of visits where each species was recovered from broth but not direct-plating (enrichment) as a fraction of visits where the species was present

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Spn</th>
<th>Hflu</th>
<th>Mcat</th>
<th>SA</th>
<th>Hpi</th>
<th>AHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW Healthy</td>
<td>4.3</td>
<td>7.3</td>
<td>3.4</td>
<td>53.6</td>
<td>4.7</td>
<td>32.7</td>
</tr>
<tr>
<td>NW Sick</td>
<td>3.5</td>
<td>2.8</td>
<td>5.0</td>
<td>67.3</td>
<td>5.9</td>
<td>38.3</td>
</tr>
<tr>
<td>MEF Sick</td>
<td>10.2</td>
<td>4.4</td>
<td>13.5</td>
<td>44.4</td>
<td>15.4</td>
<td>15.9</td>
</tr>
</tbody>
</table>
wash solution thereby largely bypassing the anterior nares. SA seems to be quite a unique pathogen in terms of its relative strength when competing with other bacteria organisms in broth. While we only isolated SA in 94 out of 3442 visits (3 %), SA out-competed respiratory bacterial pathogens in ~75 % of samplings. Previous studies have shown that SA is susceptible to hydrogen peroxide produced by other bacteria (most notably Spn, S. viridans and other Streptococcus species) [24, 25]; however in broth when SA was paired with Spn (nine cases) or AHS (25 cases) in all but one instance SA predominated. This may suggest that broth-enrichment overcomes the effect of production of H_2O_2.

Hflu and Mcat did not compete strongly with one another during broth-enrichment. However, AHS tended to predominate over all otopathogens after broth-enrichment, especially when samples were taken at healthy visits. This suggests that the broth we used for enrichment was selective for non-pathogenic normal flora.

In conclusion, we recommend using direct-plating of NP samples for most circumstances when the laboratory is asked to identify potential bacterial respiratory pathogens. Only if it is essential to identify every possible potential bacterial respiratory pathogen would broth-enrichment be of value, accepting that the technical time and effort would be material. In contrast, we recommend broth-enrichment of MEF samples because the process results in significantly improved recovery of bacterial respiratory pathogens with marginal additional technical time because other flora are infrequently present in the inocula.

Fig. 2. Tendency of different species to predominate in NWB samples when both were present in NW in healthy and AOM visits. Bars depict 95 % confidence interval (CI) for recovery of one species but not the other from broth in samples collected during health and AOM, and the star sign (*) shows if there is significant difference between healthy and AOM visits. (*P<0.05; **P<0.01; Pearson’s chi-square test.)

Funding information
This work was supported by the National Institute on Deafness and Other Communication Disorders, National Institutes of Health (grant R01-08671).

Acknowledgements
We thank all the staff at Legacy Pediatrics and Research Institute and the children and parents who agreed to participate in this study.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Written informed parental consent was obtained prior to study procedures as approved by the University of Rochester IRB and subsequently by the Rochester General Hospital IRB.

References


