Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Diagnostic Microbiology and Infectious Disease 85 (2016) 125–130

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Detection of respiratory pathogens in pediatric acute otitis media by PCR and comparison of findings in the middle ear and nasopharynx

Svetlana Yatsyshina [a], Nikolay Mayanskiy [b,c,*], Olga Shipulina [a], Tatiana Kulichenko [b,c], Natalia Alyabieva [b], Lyubovj Katosova [b], Anna Lazareva [b], Tatyana Skachkova [a], Maria Elkina [a], Svetlana Matosova [a], German Shipulin [a]

[a] Central Research Institute for Epidemiology of Rospotrebnadzor (CRIE), 111123, Novogireyevskaya Str., 3A, Moscow, Russia
[b] Scientific Center for Children's Health, 119991, Lomonosovskiy avenue, 2, Moscow, Russia
[c] I.M. Sechenov First Moscow State Medical University, 119991, B. Pirogovskaya Str., 2/4, Moscow, Russia

ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form 22 January 2016
Accepted 7 February 2016
Available online 11 February 2016

Keywords:
Acute otitis media
Middle ear fluid
Nasopharynx
Quantitative PCR
Streptococcus pneumoniae
Respiratory viruses

ABSTRACT

We conducted a series of polymerase chain reactions (PCRs) in order to detect bacteria (7 species) and viruses (17 species) in middle ear fluid (MEF) and nasopharynx (Nph) of children with acute otitis media (AOM; n = 179). Bacterial and viral nucleic acids were detected in MEF of 78.8% and 14.5% patients, respectively. The prevalence was as follows: *Streptococcus pneumoniae*, 70.4%; *Haemophilus influenzae*, 17.9%; *Staphylococcus aureus*, 16.8%; *Streptococcus pyogenes*, 12.3%; *Moraxella catarrhalis*, 9.5%; rhinovirus, 9.5%; and adenovirus, 3.4%. The overall rate of PCR-positive specimens for bacterial pathogens was 2.6 times higher, compared to culture results. The rate of PCR-positive results and the distribution of pathogens in the Nph were similar to those in the MEF. Nph PCR results had variable positive predictive values and high negative predictive values in predicting MEF findings. Our results indicate that Nph PCR could be a practical tool for examining respiratory pathogens in children with acute infections.

1. Introduction

Acute otitis media (AOM) is one of the most common pediatric infections with high rate of medical visits and antibiotic prescriptions (Lieberthal et al., 2013). Bacterial species, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pyogenes*, predominate among AOM pathogens, being responsible for 80–90% of etiologically verified cases (Vergison, 2008). Respiratory viruses like human rhinovirus (hRV), adenovirus (AdV), respiratory syncytial virus (RSV), and some others are occasionally found in the middle ear; however, their role as independent (primary) AOM-causing agents remains under discussion (Chonmaitree et al., 2012; Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2015; Pitkäranta et al., 1998; Ruohola et al., 2006; Vergison, 2008). Nevertheless, pathogenesis of AOM in children is clearly related to acute respiratory viral infections (ARVI) that, by various means, facilitate translocation of pathogen-containing secretions from inflamed nasopharynx into the middle ear (Lieberthal et al., 2013). In many cases, ARVI precede AOM, and the latter is considered as a complication of ARVI (Chonmaitree et al., 2008).

Traditionally, bacterial or viral culturing of the middle ear fluid (MEF) has been used for establishing etiology of AOM ensuring a 30–80% positive rate (Chonmaitree, 2000; Lieberthal et al., 2013). Cultural analysis depends on viable microorganisms; it has limited sensitivity and is laborious and time consuming. Principal bacterial otopathogens, *S. pneumoniae* and *H. influenzae*, belong to fastidious microorganisms with special demands for plating time and culture medium that may preclude the proper isolation of these species (Ueyama et al., 1995). The advent of molecular methods like PCR has increased the prevalence of positive MEF findings for bacterial pathogens and placed several new viruses among potential AOM pathogens (Bulut et al., 2007; Nokso-Koivisto et al., 2004; Pitkäranta et al., 1998; Ruohola et al., 2006; Ueyama et al., 1995; Williams et al., 2006; Yano et al., 2009).

Systematic use of tympanocentesis is not advocated in the current clinical practice (Lieberthal et al., 2013). This restricts availability of MEF for monitoring etiology of nonperforated AOM and implies Nph secretions as a potential proxy for MEF. Indeed, middle ear and nasopharynx findings have been compared in a number of studies, which indicated potential usefulness of nasopharynx examination (Radzikowski et al., 2011; van Dongen et al., 2013 and references therein). Most of the studies employed PCR only for virus detection using culturing for bacterial isolation and identification.

In the present work, we used a series of multiplex real-time PCRs for detecting 7 bacterial and 17 viral species in MEF of children with AOM

* Corresponding author. Tel.: +7-499-134-02-18; fax: +7-499-134-70-01.
E-mail address: mayansky@nczd.ru (N. Mayanskiy).

http://dx.doi.org/10.1016/j.diagmicrobio.2016.02.010
0732-8893/

and compared PCR results with culturing for selected bacterial pathogens. Moreover, we assessed the value of PCR findings in the nasopharynx for prediction of the pathogen presence in MEF during AOM.

2. Materials and methods

2.1. Study population and clinical specimens

This retrospective study was performed using biological specimens obtained from children below 5 years of age with AOM between August 2011 and April 2013 in 4 pediatric hospitals in Moscow, Russia, and included children who were diagnosed with AOM by an otolaryngologist and had an MEF specimen obtained from them (Mayanskiy et al., 2015). A diagnosis of AOM was defined by otolaryngologists when a patient had acute illness lasting ≤7 days with signs and physical symptoms consistent with AOM, including earache, tugging at the ear, fever, redness, and bulging of tympanum. Patients who received an antibiotic prior to enrollment or had spontaneous tympanum perforation were excluded from the present study. MEF specimens were collected by otolaryngologists after tympanocentesis and swabbing the tympanic cavity with an eSWAB kit (Copan, Italy). This procedure was performed after removal of debris and cleaning of external auditory canal (lidocaine solution with ethyl alcohol, exposure for 1 minute). Each patient contributed 1 AOM case to the study. In bilateral AOM cases, MEF specimens were obtained from both ears. At the same visit, Nph swabs were collected by means of the eSWAB kit.

Biological samples and all data were collected according to the guidelines of the hospitals' ethics committee.

All specimens were delivered to the laboratory at the Scientific Center for Children's Health (Moscow) and processed there within 24–48 hours after sampling; before cultural analyses, the specimens were stored at room temperature in a dark place. Bacterial pathogens including *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. pyogenes*, and *Staphylococcus aureus* were cultured and identified as described earlier (Mayanskiy et al., 2015). A specimen was regarded culture positive if either of these bacteria alone or in combination was isolated.

2.2. PCR for bacterial and viral respiratory pathogens

The remaining specimens were frozen and stored at −70 °C for further use in molecular assays that were performed in 2014 at the Central Research Institute for Epidemiology, Moscow, Russia, using reagent kits manufactured at this same institution (Supplement). All kits are cleared for in vitro diagnostic purposes. Total DNA and RNA were extracted from a 100-μL aliquot using the RIBO-prep nucleic acid extraction kit and eluted into the same volume of an elution buffer. cDNA was prepared by reverse transcription of 30 μL of RNA with the REVERTA-L RT kit. For PCRs, 10 μL of DNA or cDNA was used. Real-time PCR was utilized for detecting bacterial and viral nucleic acids (NAs) on a Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia). DNA from *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, *S. aureus*, and *M. catarrhalis* was detected in a quantitative manner. *S. pyogenes* and *S. aureus* were analyzed using the AmpliSens® Streptococcus pyogenes-screen-titre-FRT PCR kit and the AmpliSens® MRSA-screen-titre-FRT PCR kit, respectively. For detection of the remaining bacteria, we used the *S. pneumoniae Spn9802* gene fragment (Abdeldaim et al., 2010), the *H. influenzae hpd* gene (Theodore et al., 2012), and the *M. catarrhalis copB* gene (Greiner et al., 2003). Analytical sensitivity of these reactions was at least 5 copies of DNA per PCR (or 500 copies/mL) for every target (see Supplement for details). A real-time PCR result was considered positive when ≥1000 or 3 lg copies/mL of DNA were found.

NAs from the rest of respiratory pathogens were detected in a qualitative manner. Identification of the *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* DNA was done by the AmpliSens *M. pneumoniae*/*C. pneumoniae*-FRT PCR kit. Viral NA identification included influenza viruses A and B (using the AmpliSens Influenza virus A/B-FRT PCR kit); enterovirus (using the AmpliSens Enterovirus-FRT PCR kit); RSV, types A and B; human metapneumovirus; parainfluenzavirus, types 1–4; coronavirus OC43, E229, NL63, and HKU1; hRV; AdV, types B, C, and E; and human bocavirus (all using the AmpliSens ARVI-screen-FRT PCR kit). The Influenza virus C *HE* gene was detected by a 2-step real-time RT-PCR (see Supplement for details). Analytical sensitivity of each viral NA PCR test was at least 10 copies of DNA/cDNA per PCR (or 1000 copies/mL) for every target.

2.3. Statistics

The statistical analysis was performed using IBM SPSS Statistics for Windows, version 18.0 (IBM, Armonk, NY, USA). DNA load in different specimens was compared by the Mann–Whitney test that was considered statistically significant at $P < 0.05$. Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) of an Nph PCR result in predicting the presence of NA from a particular respiratory pathogen in MEF were calculated using an MEF specimen PCR result as the reference. Cramer's V test was used as a measure of strength of association between PCR results in MEF and nasopharynx for a particular pathogen. This test may have values from 0 to 1; higher values indicate a stronger association (Grjibovski, 2008).

3. Results

3.1. PCR analysis of MEF specimens

PCR was performed in a total of 216 MEF specimens obtained from 179 children with AOM (142 patients had unilateral AOM; 37 patients had bilateral AOM, each contributed an MEF specimen from both ears); the median patient age was 30 months (range, 1 month to 5 years). The NA from at least 1 pathogen under study (e.g., PCR-positive results) was discovered in 93.3% (167/179) children. Overall, the bacterial pathogen DNA alone had 78.8% (141/179) patients. DNA from a single species was found in 54.2% (97/179) patients, whereas DNA from 2 species was found in 20.1% (36/179) patients, and 4.5% (8/179) children had DNA from 3 to 4 species.

The most prevalent organism among pathogens under study was *S. pneumoniae*; the pneumococcal DNA was found in 70.4% (126/179) patients (Table 1). A noticeable prevalence was found for the *S. aureus* DNA, which was detected in 16.8% (30/179) patients. However, the *S. aureus* DNA was always present in a combination with NA from other pathogens, and no patients had the *S. aureus* DNA alone in the MEF (Table 1). This was in contrast with, for instance, the pneumococcal DNA that was the only NA found in 58% (73/126) *S. pneumoniae* DNA-positive patients. DNA from *M. pneumoniae* or *C. pneumoniae* was not found in MEF of any patient.

In paired MEF specimens obtained from children with bilateral AOM (n = 37), PCR results almost completely agreed. Only in 3 patients DNA from *H. influenzae* (n = 1), RNA from hRV (n = 1), and a mixture of NA from *S. aureus* and hRV (n = 1) was discovered in 1 ear in addition to the *S. pneumoniae* DNA found in both ears.

Viral NA was present in 14.5% (26/179) children, although only 2.2% (4/179) patients had viral NA alone that represented hRV (n = 3) and AdV (n = 1) (Table 1). A mixture of bacterial and viral NA was observed in 12.3% (22/179) children. The highest discovery rate was registered for the hRV RNA and the AdV DNA that were detected in MEF of 9.5% (17/179) and 3.4% (6/179) children, respectively (Table 1). NA from RSV; influenza virus, type C; and human bocavirus were found rarely (each in 1 or 2 patients) and exclusively in combination with DNA from a bacterial pathogen. No NA from influenza virus, types A and B; parainfluenzavirus; human metapneumovirus; enterovirus; and coronavirus was found in the MEF.

Table 1
Detection rate and combination prevalences of respiratory pathogens in MEF of 179 patients with AOM by real-time PCR.

Pathogen	Overall, n (%)	Combination prevalence, n (%)[a]										
		S. pneumoniae	*H. influenzae*	*S. pyogenes*	*S. aureus*	*M. catarrhalis*	hRV	AdV	RSV	InfC	HBoV	3–4 pathogens
S. pneumoniae	126 (70.4)	**73 (58%)**	8	4	14	4	5	3		2	1	12
H. influenzae	32 (17.9)	8	**11 (34%)**	1	1				1			10
S. pyogenes	22 (12.3)	4	1	**9 (41%)**	3							5
S. aureus	30 (16.8)	14	1	3	**- (0%)**	1	1					10
M. catarrhalis	17 (9.5)	4			1	**4 (24%)**	1					7
hRV	17 (9.5)	5			1	1	**3 (18%)**					7
AdV	6 (3.4)	3						**1 (17%)**				2
RSV	1 (0.6)		1						**- (0%)**			
Inf C	2 (1.1)	2								**- (0%)**		
HBoV	2 (1.1)	1									**- (0%)**	1

The sum of overall proportions of pathogens exceeded 100% because, in some patients, a combination of 2–4 pathogens was recovered. AdV = adenovirus (including types B, C, and E); InfC = influenza virus, type C; HBoV = human bocavirus; RSV = respiratory syncytial virus (including types A and B).
[a] Each value in boldface indicates the number of patients with a corresponding pathogen detected singly in the MEF; the percentages (in brackets) represent a ratio of singly detected pathogen to the overall prevalence of this pathogen.

3.2. Comparison of PCR and culturing for detection of bacterial pathogens in the MEF

Next, we compared PCR and culturing results of bacterial pathogens considering the specimen level (Table 2). Bacterial DNA was found in 91.7% (198/216) MEF specimens, whereas culture-positive results were observed in 35.7% (77/216) specimens. Thus, the overall rate of PCR-positive specimens for bacterial pathogens was 2.6 times higher (95% confidential interval [CI] 2.3–3.0 times).

The difference in the detection rate between PCR and culturing had a pathogen-specific manner. The biggest gap was observed for *H. influenzae* that was found 10 times (95% CI 6.5–138 times) more often by PCR than by culturing. For the rest of bacterial pathogens under study, the ratio of PCR-positive/culture-positive specimens varied between 2 and 5.1 (Table 2).

The overall median DNA load in culture-positive/PCR-positive MEF specimens was close to 6 lg copies/mL for all bacterial pathogens except *M. catarrhalis*, which had a median DNA load of 4.83 lg copies/mL (Table 2). The median DNA concentration of *S. pneumoniae*, *S. pyogenes*, and *S. aureus* was significantly higher (at least 1 lg copies/mL, i.e., 10 times) in culture-positive/PCR-positive specimens. In culture-positive specimens, the minimum load of the *S. aureus* DNA was 4.89 lg copies/mL; this value was 1–1.5 lg copies/mL higher comparing to the other bacteria (Table 2).

3.3. The value of Nph PCR results in predicting the presence of a pathogen in the middle ear

A Nph specimen was obtained from all patients enrolled to the study (n = 179). The overall rate of PCR-positive Nph specimens was 91.6% (164/179). Bacterial DNA was detected in 69.3% (124/179) specimens. In 1 patient, the *M. pneumoniae* DNA was detected in association with DNA from *S. pneumoniae*. None of the patients carried DNA from *C. pneumoniae* in the nasopharynx. The median load of DNA in Nph and MEF specimens was similar for all bacterial pathogens except *M. catarrhalis* that was approximately 1 lg copies/mL more abundant in the nasopharynx (Table 4). Viruses were detected in 22.3% (40/179) Nph specimens. The highest prevalence was found for hRV (8.9%) and AdV (5%) (Table 3). NAs from influenza virus B, metapneumovirus, coronavirus, and enterovirus were not detected in the nasopharynx.

The strength of agreement between PCR results in paired MEF and Nph specimens was assessed using the Cramer's V test (Grjibovski, 2008). A strong association between MEF and Nph PCR results (V > 0.6) was observed for *S. pneumoniae*, *H. influenzae*, and AdV (Table 3). The remaining microorganisms demonstrated a substantial proportion of discordant PCR results, having lower V-values (Table 3).

Next, we analyzed predictive value of Nph PCR results for the presence of NA from a certain microorganism in the MEF. A positive Nph PCR result had modest to low PPV, except for *S. pneumoniae*. Recovering the pneumococcal DNA in the nasopharynx increased a chance of positive PCR result for the pneumococcal DNA in the MEF up to 92% (PPV, Table 3). The lowest V-value and PPV were obtained for *M. catarrhalis* (0.33 and 32%, respectively) and *S. aureus* (0.42 and 46%, respectively).

The specificity and NPV of Nph PCR findings were high (Table 3). For instance, the NPV of a negative Nph result for the pneumococcal DNA was 79%. This indicated that a chance of finding this DNA in the MEF was reduced from 70.4% (a value corresponding to the prevalence in MEF; see Table 1) to 21% (i.e., 100% − NPV), if it was not present in the nasopharynx.

4. Discussion

4.1. Etiology of AOM

In the present study, we performed PCR analysis for a broad range of respiratory pathogens in MEF and Nph specimens obtained from children with AOM. To our knowledge, this is the first comprehensive

Table 2
Comparison of PCR and culturing for detection of bacterial pathogens in 216 MEF specimens.

Pathogen	PCR-positive, n (%)	Culture-positive[a], n (%)	PCR positive/culture positive, ratio (95% CI)	DNA load (lg copies/mL), median (min–max)		
				Culture positive	Culture negative	*P*[b]
S. pneumoniae	148 (68.5)	49 (22.7)	3.0 (2.6–3.6)	5.94 (3.69–8.39)	4.65 (3.01–7.57)	<**0.001**
H. influenzae	41 (18.9)	4 (1.9)	10.0 (6.5–138)	6.09 (4.00–7.22)	5.20 (3.03–6.98)	0.28
S. pyogenes	28 (13.0)	14 (6.5)	2.0 (1.8–2.7)	5.72 (3.00–8.14)	4.38 (3.04–6.74)	**0.015**
M. catarrhalis	17 (7.9)	4 (1.9)	4.2 (3.1–43)	4.83 (3.04–5.12)	5.13 (3.04–6.00)	0.62
S. aureus	31 (14.4)	6 (2.8)	5.1 (3.8–16.2)	5.96 (4.89–8.36)	4.10 (3.14–5.21)	**0.016**

[a] All culture-positive specimens were PCR-positive for the corresponding pathogen.
[b] *P* value indicates a significance of differences between DNA load in culture-positive and culture-negative specimens for the corresponding pathogen.

Table 3
The value of PCR results from the nasopharynx in predicting the presence of a pathogen in the middle ear.

Pathogen	Overall in the nasopharynx, n (%)	Paired MEF/Nph specimens, n				Sensitivity, %	Specificity, %	PPV, %	NPV, %	Cramer's V
		MEF+/Nph+	MEF−/Nph+	MEF+/Nph−	MEF−/Nph−					
S. pneumoniae	125 (69.8)	115	10	11	43	91 (86–96)	81 (70–92)	92 (87–97)	79 (69–90)	0.72 (0.61–0.83)
H. influenzae	42 (23.5)	27	15	5	132	84 (72–97)	90 (85–95)	64 (50–79)	96 (93–99)	0.67 (0.53–0.80)
S. pyogenes	39 (10.6)	10	9	12	148	45 (25–66)	94 (91–98)	53 (30–75)	93 (88–97)	0.42 (0.20–0.62)
M. catarrhalis	28 (15.6)	9	19	8	143	53 (29–77)	88 (83–93)	32 (15–49)	95 (91–98)	0.33 (0.12–0.52)
S. aureus	19 (21.8)	18	21	12	128	60 (42–78)	86 (80–91)	46 (31–62)	91 (87–96)	0.42 (0.24–0.58)
hRV	16 (8.9)	9	7	8	155	53 (29–77)	96 (93–99)	56 (32–81)	95 (92–98)	0.50 (0.26–0.73)
AdV	9 (5.0)	6	3	0	170	100	98 (96–100)	67 (36–97)	100	0.81 (0.57–1.0)

Included are pathogens that had a prevalence ≥5%. Values in brackets in columns sensitivity, specificity, PPV, NPV, and Cramer's V indicate 95% CI.

study enrolling a significant number of patients that elaborates on etiology of AOM, using parallel PCR in the MEF and nasopharynx. Comparison of PCR with culturing demonstrated a higher sensitivity of the molecular method in detecting bacterial pathogens. The increased sensitivity of PCR may be attributed, at least in part, to the fact that PCR does not depend on bacterial viability. This is of special importance for fastidious otopathogens like *S. pneumoniae* and *H. influenzae*. Our results corresponded to an earlier report demonstrating that, in patients with otitis media, PCR was more sensitive than the conventional culture method in detecting MEF *H. influenzae* by at least 5 times (Ueyama et al., 1995).

Another explanation for culture negative/PCR-positive results could be the presence of nonculturable bacteria remaining in MEF after previous AOM episode (Palmu et al., 2004). However, nonviable bacteria or DNA from dead bacteria may reside in the MEF no longer than a few days, being efficiently eliminated from the middle ear (Post et al., 1996). Instead, MEF-detectable DNA may originate from the nasopharynx through a continuous feed of the DNA-containing media. Moreover, the presence of bacterial biofilms has been proposed as a reason for culture failure but positive PCR results (Ehrlich et al., 2002; Palmu et al., 2004).

Our PCR analyses did not support the etiological role in AOM for atypical bacteria like *M. pneumoniae* and *Chlamydophila* spp., which were not detected in MEF of any patient. This is corroborated by previous reports that never demonstrated the presence of *M. pneumoniae* in the MEF (Pitkäranta et al., 2006; Rosenblut et al., 2001) and only occasionally found *Chlamydophila* spp. in AOM patients, mainly during epidemic elevations of its prevalence (Block et al., 1997; Falck et al., 1998).

Reportedly, the prevalence and distribution of viruses in MEF have not been consistent, varying in different study settings and geographic regions and having seasonal and detection method-dependent fluctuations. In several reports from Finland, detection rate of viruses in the MEF was 48–67%, with the predominance of picornaviruses (hRV and enteroviruses) (Nokso-Koivisto et al., 2004; Pitkäranta et al., 1998; Ruohola et al., 2006). A study from the United States identified 41% virus-positive AOM patients, of whom 75% had RSV in the MEF (Heikkinen et al., 1999). In a Turkish report, a respiratory virus was found in MEF of 32.5% AOM patients; RSV and hRV had the highest identification rate (46.5% and 25.6% among virus-positive patients, respectively) (Bulut et al., 2007). The virus detection rate was much lower in a paper from Japan that identified respiratory viruses in 9.3% of AOM patients by culturing; almost a half of detected viruses were represented by RSV (Yano et al., 2009). Similar results were observed in a study from Chile, where only 13% of children with AOM had a virus in the MEF (Rosenblut et al., 2001).

In our patient cohort, only hRV and AdV had a somewhat appreciable representation in the MEF, covering collectively more than 85% (17/26) virus-positive patients. RSV that has been consistently detected as one of the most abundant viruses in MEF (Patel et al., 2007) was found only in 1 patient (in combination with *H. influenzae*). This finding corresponded to a low incidence of the RSV infection during the study period in Russia that was estimated as 2–10% of all acute respiratory infection visits (Iatsyshina et al., 2013).

The role of respiratory viruses in development of AOM has been discussed in several interesting papers (Chonmaitree et al., 2012; Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2015). Clinically evident ARVI has been shown to associate with increased risk of AOM, involving a wide range of mechanisms. Although the significance of viruses in development of AOM has been suggested in a chinchilla model in vivo (Bakaletz et al., 1993), their importance as independent etiological agents remains controversial. A viral infection facilitates the passage of bacterial pathogens from the nasopharynx into the middle ear, supporting progression to clinical illness (AOM) (Lieberthal et al., 2013). Moreover, respiratory viruses, particularly hRV, promote spreading of bacterial agents among the human population and may produce an imbalance of the Nph microbiota enhancing proliferation of *S. pneumoniae* or *H. influenzae* there (Heikkinen and Chonmaitree, 2003). Similarly, our present results do not favor the direct role of respiratory viruses in development of AOM demonstrating, in the majority of patients with AOM, low prevalence of viruses in conjunction with the presence of significant bacterial otopathogens in the MEF or nasopharynx.

Table 4
Bacterial DNA load in Nph and MEF specimens.

Bacterial pathogen	DNA load (lg copies/mL), median (min–max)		*P* value
	Nph (n = 179)	MEF (n = 216)	
S. pneumoniae	5.1 (3.0–8.9)	5.1 (3.0–8.4)	0.694
H. influenzae	5.1 (3.1–8.2)	5.0 (3.0–7.2)	0.649
S. pyogenes	4.8 (3.1–8.3)	5.2 (3.0–8.1)	0.449
M. catarrhalis	5.5 (3.7–7.3)	4.4 (3.0–6.0)	**0.01**
S. aureus	4.9 (3.4–8.5)	4.5 (3.2–8.4)	0.266

The number of each bacterial pathogen isolate tested is indicated in Table 2 for MEF specimens (the column "PCR positive") and in Table 3 for Nph specimens (the column "Overall in the nasopharynx").

4.2. Predictive value of PCR results in the nasopharynx for the assessment of MEF

The PPV of Nph specimen examination by PCR discovered a considerable, pathogen-dependent variability. The highest PPV (92%) was found for *S. pneumoniae*, the most prevalent pathogen. *S. aureus* and *M. catarrhalis* demonstrated low PPVs and weak association between MEF and Nph PCR results, having a high proportion of MEF-negative/Nph-positive results. Remarkably, the *S. aureus* DNA was always detected in a combination with the other NAs, and the *M. catarrhalis* DNA was more prevalent in the nasopharynx than in the middle ear. These data may suggest that both species are more likely to colonize the

nasopharynx than to serve as the primary cause of clinical infection in the middle ear. Thus, in general, Nph PCR results were more reliable in excluding the presence of a respiratory pathogen NA in the MEF. Consistently high NPVs suggested that, if an Nph PCR result was negative for a particular NA, this NA was unlikely to be detected in MEF of an AOM patient.

Of note, the overall concordance of MEF and Nph PCR results, i.e., the proportion of specimens with the same PCR results both in the nasopharynx and MEF, was high (range, 82–98%). However, the diagnostic significance of such an agreement was limited due to a large number of negative concordant results that the least prevalent pathogens, like viruses, had.

Quantitative real-time PCR and multiplex detection of five bacterial species have not been used for assessment of association between MEF and Nph microbiota in AOM patients before. Most studies evaluating concordance between MEF and Nph findings have used conventional culture, whereas PCR was employed in only 2 papers for analysis of a narrow set of species (Eser et al., 2009; Ueyama et al., 1995; van Dongen et al., 2013). Overall, results reported in the literature demonstrate modest to low PPVs (around 50%) for the most relevant microbes, but moderate to high NPVs, ranging from 68% to 97% (van Dongen et al., 2013). In the present study, we observed higher PPVs for *S. pneumoniae* and *H. influenzae* than has previously been reported, which suggests better performance of the PCR method in predicting MEF findings.

Some limitations of the present study need to be mentioned. First, the patient cohort included children who required tympanocentesis. This may have biased our sample toward a more severe AOM, thus disturbing the pathogen distribution and increasing the proportion of detected bacteria at the expense of viruses. Next, we did not examine patients with spontaneous otorrhea who may have a more aggressive AOM course associated with bacteria like *S. pyogenes*. Finally, swabbing the tympanic cavity after tympanocentesis could have been less effective comparing to aspiration of the MEF in terms of collecting detectable amount of viral pathogens.

5. Conclusions

Taken together, our results indicate that multiplex real-time PCR is a reasonable approach for detection of respiratory pathogens in patients with AOM. Using MEF specimens as the reference, we demonstrated that the parallel PCR examination of Nph specimens provided useful information on MEF pathogens that had a high predictive value for excluding a pathogen. Investigation of the Nph microbiota could be a reliable proxy for MEF examination in epidemiological studies and in trials looking for potential benefits of new vaccines for respiratory pathogens. In addition, PCR analysis of Nph specimens could be helpful in predicting bacterial complications during ARVI, for instance, AOM.

In conclusion, further implementation of sensitive, rapid, and multiplexed molecular methods would extend our understanding of the respiratory pathogen interplay and improve diagnostics of respiratory infections.

Conflicts of interest

None.

Acknowledgements

We thank Dr A. Ivanenko for his help in collecting patient specimens. Excellent technical assistance of Dr O. Ponomarenko is appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2016.02.010.

References

Abdeldaim GMK, Strålin K, Korsgaard J, Blomberg J, Welinder-Olsson C, Herrmann B. Multiplex quantitative PCR for detection of lower respiratory tract infection and meningitis caused by Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitides. BMC Microbiol 2010;10:310. [http://www.biomedcentral.com/1471-2180/10/310].

Bakaletz LO, Daniels RL, Lim DJ. Modeling adenovirus type 1-induced otitis media in the chinchilla: effect on ciliary activity and fluid transport function of eustachian tube mucosal epithelium. J Infect Dis 1993;168:865–72.

Block SL, Hammerschlag MR, Hedrick J, Tyler R, Smith A, Roblin P, et al. Chlamydia pneumonia in acute otitis media. Pediatr Infect Dis J 1997;16:858–62.

Bulut Y, Güven M, Otlu B, Yenişehirli G, Aladağ I, Eyibilen A, et al. Acute otitis media and respiratory viruses. Eur J Pediatr 2007;166(3):223–8. [Epub 2006 Sep 12. PubMed PMID: 16967296].

Chonmaitree T. Viral and bacterial interaction in acute otitis media. Pediatr Infect Dis J 2000;19:S24–30.

Chonmaitree T, Revai K, Grady JJ, Clos A, Patel JA, Nair S, et al. Viral upper respiratory tract infection and otitis media complication in young children. Clin Infect Dis 2008;46(6):815–23. http://dx.doi.org/10.1086/528685.

Chonmaitree T, Ruohola A, Hendley JO. Presence of viral nucleic acids in the middle ear: acute otitis media pathogen or bystander? Pediatr Infect Dis J 2012;31(4):325–30. http://dx.doi.org/10.1097/INF.0b013e318241afe4.

Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA 2002;287:1710–5.

Eser OK, Ipci K, Alp S, Akyol U, Unal OF, Hascelik G, et al. Efficacy of nasopharyngeal culture in identification of pathogen in middle ear fluid in chronic otitis media with effusion. Indian J Med Microbiol 2009;27(3):237–41. http://dx.doi.org/10.4103/0255-0857.53206. [PubMed PMID: 19584505].

Falck G, Engstrand I, Gnarpe J, Gnarpe H. Association of Chlamydia pneumoniae with otitis media in children. Scand J Infect Dis 1998;30:377–80.

Greiner O, Day PJR, Altwegg M, Nadal D. Quantitative detection of Moraxella catarrhalis in nasopharyngeal secretions by real-time PCR. J Clin Microbiol 2003;41(4):1386–90.

Grjibovski AM. Analysis of nominal data (independent observations). Hum Ecol 2008;6:58–68.

Heikkinen T, Chonmaitree T. Importance of respiratory viruses in acute otitis media. Clin Microbiol Rev 2003;16(2):230–41.

Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N Engl J Med 1999;340(4):260–4.

Iatsyshina SB, Konovalov AV, Magkoeva ZG, Praded MN, Shelkovskaia LP, Perevozchikova LA, et al. Laboratory diagnostics in evaluation of acute respiratory viral infection morbidity in 2010–2011 epidemic season. Zh Mikrobiol Epidemiol Immunobiol 2013;1:33–8.

Lieberthal AS, Carroll AE, Chonmaitree T, Ganiats TG, Hoberman A, Jackson MA, et al. The diagnosis and management of acute otitis media. Pediatrics 2013;131(3):e964–99. http://dx.doi.org/10.1542/peds.2012-3488.

Mayanskiy N, Alyabieva N, Ponomarenko O, Pakhomov A, Kulichenko T, Ivanenko A, et al. Bacterial etiology of acute otitis media and characterization of pneumococcal serotypes and genotypes among children in Moscow, Russia. Pediatr Infect Dis J 2015;34(3):255–60. http://dx.doi.org/10.1097/INF.0000000000000554. [PubMed PMID: 25232779].

Nokso-Koivisto J, Räty R, Blomqvist S, Kleemola M, Syrjänen R, Pitkäranta A, et al. Presence of specific viruses in the middle ear fluids and respiratory secretions of young children with acute otitis media. J Med Virol 2004;72(2):241–8. [PubMed PMID: 14695665].

Nokso-Koivisto J, Marom T, Chonmaitree T. Importance of viruses in acute otitis media. Curr Opin Pediatr 2015;27(1):110–5. http://dx.doi.org/10.1097/MOP.0000000000000184.

Palmu AA, Saukkoriipi PA, Lahdenkari MI, Kuisma LK, Makela PH, Kilpi TM, et al. Does the presence of pneumococcal DNA in middle-ear fluid indicate pneumococcal etiology in acute otitis media? J Infect Dis 2004;189(5):775–84. [Epub 2004 Feb 16].

Patel JA, Nguyen DT, Revai K, Chonmaitree T. Role of respiratory syncytial virus in acute otitis media: implications for vaccine development. Vaccine 2007;25(9):1683–9. [Epub 2006 Nov 9].

Pitkäranta A, Virolainen A, Jero J, Arruda E, Hayden FG. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998;102(2):291–5.

Pitkäranta A, Roivainen M, Blomgren K, Peltola J, Kaijalainen T, Räty R, et al. Presence of viral and bacterial pathogens in the nasopharynx of otitis-prone children. A prospective study. Int J Pediatr Otorhinolaryngol 2006;70(4):647–54. [Epub 2005 Sep 27. PubMed PMID: 16198005].

Post JC, Aul JJ, White GJ, Wadowsky RM, Zavoral T, Tabari R, et al. PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media. Am J Otolaryngol 1996;17:106–11.

Radzikowski A, Skorka A, Mikolajczyk W. Does bacterial flora predict etiology of acute otitis media in children? Pediatr Pol 2011;86:620–3.

Rosenblut A, Santolaya ME, Gonzalez P, Corbalan V, Avendano LF, Martinez MA, et al. Bacterial and viral etiology of acute otitis media in Chilean children. Pediatr Infect Dis J 2001;20(5):501–7. [PubMed PMID: 11368107].

Ruohola A, Meurman O, Nikkari S, Skottman T, Salmi A, Waris M, et al. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin Infect Dis 2006;43:1417–22.

Theodore MJ, Anderson RD, Wang X, Katz LS, Vuong JT, Bell ME, et al. Evaluation of new biomarker genes for differentiating Haemophilus influenzae from Haemophilus haemolyticus. J Clin Microbiol 2012;50(4):1422–4.

Ueyama T, Kurono Y, Shirabe K, Takeshita M, Mogi G. High incidence of Haemophilus influenzae in nasopharyngeal secretions and middle ear effusions as detected by PCR. J Clin Microbiol 1995;33(7):1835–8. [PubMed PMID: 7665655; PubMed Central PMCID: PMC228280].

van Dongen TMA, van der Heijden GJMG, van Zon A, Bogaert D, Sanders EAM, Schilder AGM. Evaluation of concordance between the microorganisms detected in the nasopharynx and middle ear of children with otitis media. Pediatr Infect Dis J 2013;32:549–52.

Vergison A. Microbiology of otitis media: a moving target. Vaccine 2008;26(Suppl 7):G5–G10.

Williams JV, Tollefson SJ, Nair S, Chonmaitree T. Association of human metapneumovirus with acute otitis media. Int J Pediatr Otorhinolaryngol 2006;70(7):1189–93. [Epub 2006 Jan 19. PubMed PMID: 16427144; PubMed Central PMCID: PMC1476701].

Yano H, Okitsu N, Hori T, Watanabe O, Kisu T, Hatagishi E, et al. Detection of respiratory viruses in nasopharyngeal secretions and middle ear fluid from children with acute otitis media. Acta Otolaryngol 2009;129:19–24.