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Running title: Pharyngeal flora in children

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SUMMARY

To improve our current understanding of normal flora in children, we investigated bacterial isolates from pharynx and nasopharynx of 173 and 233 healthy children, respectively. The bacterial isolation rates were compared among three age groups: infants (<1 year), toddlers (1–5 years), and school-age children (6–15 years). Gram-positive cocci (GPC) were the predominant bacteria in the pharynx (*Streptococcus mitis/oralis*, 87.3%; *Streptococcus salivarius*, 54.3%; *Rothia mucilaginosa*, 41.6%; *Staphylococcus aureus*, 39.3%). Among infants, *Streptococcus salivarius* and *Neisseria subflava*, which are related to the development of teeth, were significantly lower than in the other age groups ($p < 0.0001$, *S. salivarius*; $p < 0.01$, *N. subflava*). Gram-negative rods (GNR) predominated the nasopharynx (*Moraxella catarrhalis*, 32.1%; and *Moraxella nonliquefaciens*, 28.3%) except for *Corynebacterium pseudodiphtheriticum* (44.2%) of gram-positive rods. Among toddlers, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, which are the most common pathogens in acute otitis media, were significantly higher than in the infant group ($p < 0.05$ for both). Among bacterial species implicated in pediatric respiratory infection, *Streptococcus pyogenes* was isolated in 3.5% of pharyngeal samples. *S. pneumoniae* and *Haemophilus influenzae* were isolated in 22.3% and 17.2% of nasopharyngeal samples, respectively. In conclusion, normal flora of the respiratory tract differs not only by sampling site but also by age group.

INTRODUCTION

The pharynx and nasopharynx are important routes through which pathogens invade the respiratory tract and cause respiratory tract infections. Therefore, samples are often obtained from these parts. While studies and diagnostic results have reported the presence of different pathogens among normal flora (1–6), most of these reports are either outdated or focused only on specific pathogens such as *Streptococcus pneumoniae* or *Haemophilus influenzae*. Although normal flora in the pharynx changes with age, no studies performed to date have reported differences in the abundance of bacteria in the changing flora. Notably, changes in flora have been reported before and after the administration of recently approved vaccines, especially in *Streptococcus pneumoniae* or *Haemophilus influenzae*, which are the objective pathogens of vaccines that were introduced in Japan in 2008 and 2010, respectively (7,8). To improve our current understanding of the normal flora and their age-related changes in the pharynx and nasopharynx in children, we investigated the bacterial isolates from both the pharynx and nasopharynx of subjects in three different pediatric age groups.

MATERIALS AND METHODS

The study was approved by the Kawasaki Medical Center Ethics Committee, Okayama, Japan (No. 2972). Verbal informed consent was obtained from all parents.

Subjects

Children aged between 1 month and 15 years, who visited one of three clinics (Nakahama Clinic and Nakano Children's Hospital in Osaka, Japan; Satoh Children's Clinic in Niigata, Japan) for regular checkups or vaccinations from March 2013 to June 2014, were enrolled in the study. Swab samples from the pharynx were taken to the Nakahama Clinic and Nakano Children's Clinic, while those from the nasopharynx were taken to the Satoh Children's

Clinic. The origin of each sample was decided randomly. The bacterial detection rates of samples obtained from the pharynx and nasopharynx were calculated and compared among the three age groups: infants, toddlers, and school-children. An infant was defined as a child aged <1 year, a toddler as a child aged 1–5 years, and a school-child as a child aged 6–15 years.

Laboratory materials

ESwab 481C (Copan Diagnostics Inc., Brescia, Italy) was used to collect pharyngeal and nasopharyngeal samples. To avoid damage during transportation, the swabs were sealed in previously prepared duplex tubes and sent to the Kyurin Medical Laboratory at a temperature of 2 to 8°C. Three types of cell culture media were used: defibrinated sheep blood agar (Kyokuto Pharmaceutical industrial Co. Ltd, Tokyo, Japan), chocolate agar (Kyokuto Pharmaceutical industrial Co. Ltd, Tokyo, Japan), and Drigalski agar (Eiken Chemical Co. Ltd, Tokyo, Japan). All the isolates were identified by MALDI-TOF MASS system (VITEK-MS™, Biomerieux Japan), and Microbial classification identification analyzer (VITEK-2™, Biomerieux Japan). Quantitative culture with dilution was performed on each medium, after which the samples were kept at 35°C for 20–48 h. Blood agar and chocolate agar were stored in 5% CO₂. The number of colony-forming units (cfu) of the target bacteria was also counted. Bacteria detected at fewer than 100 cfu/swab were eliminated, except for *Streptococcus pneumoniae* and *Haemophilus influenzae*, which are the main pathogens responsible for pediatric respiratory infections. Furthermore, we detected three atypical pathogens of respiratory infections, including *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Borderella pertussis* using polymerase chain reaction, as described previously (9,10).

Statistical analysis

GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. The differences between the two groups were analyzed using Chi-squared test, Student-t test, Fisher's exact test, or Mann-Whitney U test, respectively (95% confidence interval). Results were expressed as mean $\pm$ standard deviation (SD). *P* values of <0.05 were considered significant.

RESULTS

Overall, 173 pharyngeal and 233 nasopharyngeal samples of healthy children were obtained. The number of samples and mean ages ($\pm$ SD) of infants, toddlers, and school-children was 132 and 0.5 ($\pm$ 0.3) years, 158 and 2.6 ($\pm$ 0.3) years, and 114 and 8.9 ($\pm$ 2.5) years, respectively. Between the two groups, the mean ages ($\pm$ SD), the ratio of subjects unvaccinated with pneumococcal conjugate vaccine (PCV) and *Haemophilus influenzae* type b vaccine in infants and toddlers were significantly higher in the nasopharynx group than in the pharynx group. There was no significant difference in the proportion of boys and history of antibiotic use between these two groups.

The bacteria isolated from the pharynx are detailed in Table 1. Gram-positive cocci (GPC) were predominant in the pharynx. *Streptococci* were isolated from all children; among the *Streptococcus* spp., *Streptococcus mitis oralis* (87.3%) and *Streptococcus salivarius* (54.3%) were the most frequently isolated species of bacteria. The isolation rate of methicillin-resistant *Streptococcus aureus* (MRSA) was merely 4.6%. Gram-negative rods (GNR), including *Moraxella catarrhalis* (27.7%), *Haemophilus influenzae* (23.7%), and *Neisseria subflava* (22.5%), were also isolated from the pharynx.

Table 2 shows the bacteria isolated from the nasopharynx of healthy children. In this cohort, GNR were predominantly observed. *Corynebacterium pseudodiphtheriticum* (44.2%), *Moraxella catarrhalis* (32.1%), and *Moraxella nonliquefaciens* (28.3%) were the most

frequently isolated species. *Streptococcus aureus* (24.0%, 1.7% of MRSA) and *Streptococcus pneumoniae* (17.2%) were the most frequently isolated GPC.

Fig. 1 reveals the isolation rates of seven predominant bacteria obtained from children's pharynx by total and age group. Among infants, *Streptococcus salivarius*, *Neisseria subflava*, and *Haemophilus influenzae* were less frequently isolated than those in other age groups. Among school-children, *Rhodotorula mucilaginosa* was more frequently isolated than that in toddlers, while *Moraxella catarrhalis* was less frequently isolated in school-children.

Fig. 2 shows the isolation rates of the seven predominant bacteria obtained from children's nasopharynx by total and age group. When compared to other age groups, the isolation rates of *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Corynebacterium pseudodiphtheriticum* among toddlers were significantly higher, while that of *Streptococcus aureus* was significantly lower.

With regards to the three atypical pathogens, none of the pathogens were isolated from the nasopharynx and pharynx except for *Chlamydia pneumoniae*. There were three children from the samples of whom *Chlamydia pneumoniae* (0.6%; 3/400) was isolated; the participants were a 4-month-old boy, a 5-year-old girl, and a 10-month-old girl.

DISCUSSION

In previous studies, common bacterial isolates from the pharynx of healthy children have generally been found to be composed of α - and β -hemolytic streptococci, staphylococci, neisseriae, diphtheroids, and others (11). However, these reports are few and outdated (12–15), and most studies are focused on particular species as opposed to describing the complete flora (12–15). Additionally, all were published before the introduction of the PCV and *Haemophilus influenzae* type b vaccines. To the best of our knowledge, the present study is the first to report total aerobic bacterial flora in the pharynx of healthy children.

GPC, especially *Streptococcus* spp. (*S. mitis oralis*, *S. salivarius*, and *S. parasanguinis*), predominated the pharynx. *S. pyogenes*, which is the most important pathogen in pharyngitis, was present in only 3.5% of healthy children. Pichichero and Casey reported that 5–10% of children were carriers for *S. pyogenes* (16). Although the rate of *S. pyogenes* carriers may be affected by changes in the incidence of *S. pyogenes* infection, our result is similar to that of previous studies.

When comparing bacteria isolated from the pharynx, *S. salivarius*, *H. influenzae*, and *N. subflava* were less frequently isolated among infants compared to other age groups. *S. salivarius* is known to be the first bacterial species to colonize dental plaques (17). Since tooth development is yet to occur in many infants, we hypothesized that the proportion of *S. salivarius* isolated from infants would be lower. *N. subflava* is a common non-pathogenic inhabitant of the human upper respiratory tract (18), and it easily forms biofilms (19) in the oral cavity and teeth, much like *R. mucilaginosa*. This may account for the more prevalent isolation in toddlers and school-children, since, unlike infants, they have teeth. Therefore, the change in bacterial flora in the pediatric pharynx might be related to teeth development.

In the nasopharynx of children, *C. pseudodiphtheriticum*, *M. catarrhalis*, and *M. nonliquefaciens* were frequently isolated. *S. pneumoniae* and *H. influenzae*, which are important pathogens of pediatric respiratory infections, were isolated in approximately 20% of healthy children. Comparing the different age groups, *S. pneumoniae*, *M. catarrhalis*, and *C. pseudodiphtheriticum* were more frequently isolated among toddlers as compared to school-children. Some studies have reported higher isolation rates of *S. pneumoniae* from the nasopharynx in children aged <7 years (1–4). *S. pneumoniae* is an important pathogen in acute otitis media (AOM) (5), which is caused by bacteria entering the middle ear from the nasopharynx. This suggests that there might be a connection between a contact with carriers of these bacteria at day care centers and the onset of AOM (20). Preschool-children are more

affected by AOM than school-children (21–22). Therefore, we can speculate that there is a reduction in the rate of viridans streptococci with the onset of AOM, which is followed by an increase in AOM-related pathogens (3). In contrast, the isolation rate of *S. aureus* was higher among school-children as compared to toddlers. Regev-Yochay et al. (23) proposed that this could be due to bacterial interference between the two isolates (*S. aureus* and *S. pneumoniae*). As invasive pneumococcal diseases may more often arise naturally in children aged <5 years, the isolation of *S. pneumoniae* may decrease in school-children, while that of *S. aureus* may increase due to a decrease in the interference by *S. pneumoniae*. Thus, a change in bacterial flora in the nasopharynx of healthy children is thought to be influenced by close contact with other children in day care centers or by their immunity to other bacterial infections. Moreover, the rate of children unvaccinated with PCV was significantly higher in schoolchildren than in other age groups, as shown in Table 3. Therefore, it is safe to speculate an absence of association between PCV and the low isolation rate of *S. pneumoniae* in school-children.

Finally, it is important to discuss atypical pathogens of respiratory infections isolated from healthy children. Studies have previously attempted to isolate each atypical respiratory pathogen from healthy people. Oishi et al. reported the prevalence of *C. pneumonia* in a nationwide surveillance in Japan (24). As mentioned in the report, the isolation rate of *C. pneumoniae* is low, even in children with respiratory infections. However, *C. pneumoniae* infection is frequently asymptomatic or only mildly symptomatic (25). Therefore, it is possible to isolate *C. pneumoniae* from healthy children, such as the subjects in the present study. Usluer and Colak investigated the isolates of *Mycoplasma pneumoniae* from a healthy population aged 8–16 years, which is the peak age for pneumonia caused by *M. pneumoniae*, and the isolation rate of *M. pneumoniae* was merely 2.5% (26). To the best of our knowledge, there are no reports describing the isolation of *Bordetella pertussis* from healthy children. In

Japan, the vaccine for *B. pertussis* has been approved for routine immunization and high levels of vaccine coverage ($\geq 95\%$ among children aged <5 years) have been reported in Japan (27). However, it was reported that acellular pertussis vaccines, which were used in many countries including Japan, protected against disease but failed to prevent infection and transmission (28). As it happens, it is possible for an outbreak of pertussis to arise even among the fully vaccinated population in Japan (9). Hence, we speculated that there was no epidemic of pertussis in the observed area during surveillance. Our results revealed the isolation of very few atypical pathogens from the pharynx of healthy children.

Six potential limitations to the present research can be identified. First, we did not obtain samples from both the pharynx and nasopharynx of the same participant. However, we strongly believe that the sample size is large enough to circumvent this limitation. Second, we did not quantify the bacteria, which could not be assessed accurately owing to the method of sample collection used. Third, because we used the culture method alone, there might be certain bacteria which we were unable to detect. Presently, there have been some reports on the analysis of the microbiome using pyrosequencing analysis of the 16S rRNA gene (29). However, we focused on comparing our data with the culture results which were used in routine medical care. Having said that, we look forward to using pyrosequencing analysis in the future. Next, we did not take into account the time of obtaining the sampling, such as seasons. We could take samples without a seasonal bias, however, number of samples in each season, especially in case of being divided into the age groups. Therefore, it was difficult to analyze differences arising due to changing seasons. It is crucial that future studies consider these limitations. Finally, we did not analyze the serotypes of *S. pneumoniae* and *H. influenzae*. Previous reports have described that almost all of both the strains detected from children's nasopharynx were of serotypes uncovered by PCV13 and *H. influenzae* type b

vaccines (30–32). Therefore, we speculate that most of the strains isolated by us were serotypes uncovered by the aforementioned vaccines.

In conclusion, normal flora of the respiratory tract differs not only by sampling site but also according to the age group. In the pharynx, GPC were predominant, and the change in flora could possibly be related to teeth development. While in the nasopharynx, GNR predominated, and the change in flora is speculated to be related to the contacts and immunity of children. We hope that the present findings lead to further analysis in other pediatric populations, with an aim to contribute to diagnostic and other clinical practices.

Conflict of interest: None to declare.

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Figure legends:

Fig. 1. Predominant bacteria isolated from the pharynx according to age group.

Fig. 2. Predominant bacteria isolated from the nasopharynx according to age group

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Table 1. Bacteria in the pharynx of healthy children

Pharynx (n=173)		
Gram-positive cocci (GPC)	No. of isolates	Isolation rate (%)
<i>Streptococcus</i> spp.	173	100
<i>S. mitis/oralis</i>	151	87.3
<i>S. salivarius</i>	94	54.3
<i>S. parasanguinis</i>	28	16.2
<i>S. pneumoniae</i>	11	6.4
<i>S. pyogenes</i>	6	3.5
<i>S. agalactiae</i>	3	1.7
<i>S. dysgalactiae</i> ssp. <i>equisimilis</i>	2	1.2
Other <i>Streptococci</i>	13	7.5
<i>Gemella haemolysans</i>	22	12.7
Other <i>Gemella</i> spp.	3	1.7
<i>Facklamia</i> spp.	0	0
<i>Staphylococcus</i> spp.	80	46.2
<i>S. aureus</i> ¹⁾	68	39.3
MRSA	8	4.6
<i>S. epidermidis</i>	16	9.3
Other <i>Staphylococci</i>	3	1.7
<i>Rothia mucilaginosa</i>	72	41.6
<i>Rothia dentocariosa</i>	5	2.9
Other GPC	0	0
Gram-Positive Rods	No.	Rate (%)
<i>Corynebacterium</i> spp.	18	10.4

<i>C. pseudodiphtheriticum</i>	10	5.8
<i>C. propinquum</i>	8	4.6
<i>C. tuberculostrictum</i>	0	0
Other <i>Corynebacterium</i>	0	0
Other GPR	0	0
Gram-Negative Cocci	No.	Rate (%)
<i>Moraxella</i> spp.	58	33.5
<i>M. catarrhalis</i>	48	27.7
<i>M. nonliquefaciens</i>	17	9.8
<i>M. lacunata/osloensis</i>	4	2.3
<i>Neisseria</i> spp.	52	30.1
<i>N. subflava</i>	39	22.5
<i>N. mucosa</i>	12	6.9
<i>N. cinerea/lactamica</i>	3	1.7
Gram-negative rods and others	No.	Rate (%)
<i>Haemophilus influenzae</i>	41	23.7
<i>H. parainfluenzae /parahaemolyticus</i>	3	1.7
<i>Escherichia coli</i>	8	4.6
Other <i>Enterobacteriaceae</i>	6	3.5
NFGNR²⁾	14	8.1
<i>Acinetobacter baumannii</i> complex	1	0.5
<i>Acinetobacter junii</i>	1	0.5
<i>Brevundimonas diminuta</i>	1	0.5
<i>Capnocytophaga ochracea</i>	1	0.5
<i>Chryseobacterium indologenes</i>	1	0.5

<i>Ochrobactrum anthropi</i>	1	0.5
<i>Pseudomonas aeruginosa</i>	1	0.5
<i>Pseudomonas fluorescens</i>	6	3.0
<i>Pseudomonas veronii</i>	2	1.0
<i>Pseudomonas viridiflava</i>	1	0.5
<i>Psychrobacter phenylpyruvicus</i>	1	0.5
Anaerobes	6	3.5
<i>Actinomyces odontolyticus</i>	4	2.0
<i>Actinomyces viscosus</i>	5	2.5
<i>Veillonella parvula</i>	2	1.0
<i>Prevotella melaninogenica</i>	1	0.5
Fungi	2	1.0
<i>Candida albicans</i>		
Atypical pathogens	No. of isolates	Isolation rate (%)
<i>Mycoplasma pneumoniae</i>	0	0
<i>Chlamydia pneumoniae</i>	1	0.6
<i>Bordetella pertussis</i>	0	0

¹⁾*S. aureus* included MRSA, Methicillin-resistant *S. aureus*; ²⁾NFGNR, Non-Fermenting

Gram-Negative Rod

Table 2. Bacteria in the nasopharynx of healthy children

Nasopharynx (n=233)		
Gram-positive cocci	No.	Rate (%)
<i>Staphylococcus</i> spp.	111	47.6
<i>S. aureus</i> ¹⁾	56	24
MRSA	4	1.7
<i>S. epidermidis</i>	56	24
Other <i>Staphylococci</i>	20	8.6
<i>Streptococcus</i> spp.	67	28.8
<i>S. pneumoniae</i>	40	17.2
<i>S. mitis/oralis</i>	14	6
<i>S. salivarius</i>	2	0.9
<i>S. parasanguinis</i>	2	0.9
<i>S. pyogenes</i>	0	0
<i>S. agalactiae</i>	0	0
<i>S. dysgalactiae</i> ssp. <i>equisimilis</i>	0	0
Other <i>Streptococci</i>	12	5.2
<i>Gemella haemolysans</i>	1	0.4
Other <i>Gemella</i> spp.	0	0
<i>Facklamia</i> spp.	3	1.3
<i>Rothia mucilaginosa</i>	3	1.3
<i>Rothia dentocariosa</i>	0	0
Other GPC	33	14.2
Gram-positive rods	No.	Rate (%)

<i>Corynebacterium</i> spp.	121	51.9
<i>C. pseudodiphtheriticum</i>	103	44.2
<i>C. propinquum</i>	19	8.2
<i>C. tuberculosteariticum</i>	5	2.2
Other <i>Corynebacterium</i>	8	3.4
Gram-negative cocci	No.	Rate (%)
<i>Moraxella</i> spp.	125	53.7
<i>M. catarrhalis</i>	75	32.1
<i>M. nonliquefaciens</i>	66	28.3
<i>M. lacunata/osloensis</i>	7	3
Other <i>Enterobacteriaceae</i>	1	0.5
NFGNR²⁾	3	1.3
<i>Acinetobacter baumannii</i> complex	1	0.4
<i>Bordetella bronchiseptica</i>	1	0.4
<i>Brevundimonas diminuta</i>	1	0.4
<i>Ochrobactrum anthropi</i>	1	0.4
<i>Pseudomonas oryzae</i>	1	0.4
<i>Pseudomonas viridiflava</i>	1	0.4
<i>Stenotrophomonas maltophilia</i>	1	0.4
Anaerobes	1	0.4
<i>Actinomyces neuii</i>	1	0.4
Fungi	1	0.4
<i>Candida albicans</i>		
Atypical pathogens	No. of isolates	Isolation rate (%)
<i>Mycoplasma pneumoniae</i>	0	0

<i>Chlamydia pneumoniae</i>	2	0.9
<i>Bordetella pertussis</i>	0	0

¹⁾*S. aureus* included MRSA; MRSA, Methicillin-resistant *S. aureus*; ²⁾NFGNR, Non-

Fermenting Gram-Negative Rod

Accepted Manuscript

Table 3. Characteristics of the enrolled healthy children

		Total (n=406)	Pharynx group*(n=173)	Nasopharynx group**(n=233)	P-value
Mean Age, years ($\pm$ SD)		3.7 $\pm$ 3.8	3.2 $\pm$ 4.0	4.1 $\pm$ 5.6	<0.0001
Proportion of boys (Number of Males/ Total)		51.0% (207/406)	53.2% (92/173)	49.3% (115/233)	0.58
History of antibiotic use ¹⁾		27.1%	24.3%	29.2%	0.27
Unvaccinated with PCV ²⁾	infants	17.4% (23/132)	9.6% (8/83) ³⁾	30.6% (15/49) ⁵⁾	0.0021
	toddlers	17.7% (28/158)	7.8% (4/51) [*]	27.3% (24/107) [*]	0.0265
	School children	62.3% (71/114)	89.2% (33/37)	87.0% (67/77)	0.5005
	total	30.2% (122/404)	26.3% (45/171)	45.4% (106/233)	<0.0001
Unvaccinated with Hib ²⁾	infants	16.7% (22/132)	7.2% (6/83) ⁴⁾	32.7% (16/49) ⁶⁾	0.0002
	toddlers	17.7% (28/158)	7.8% (4/51) [*]	27.3% (24/107) [*]	0.0265
	Schoolchildren	86.8% (99/114)	89.2% (33/37)	85.7% (66/77)	0.7706
	total	29.7% (120/404)	25.2% (43/171)	45.5% (106/233)	<0.0001

PCV, pneumococcal conjugate vaccine Hib, *Haemophilus influenzae* type b; ¹⁾History of antibiotic use was defined as subjects who had been prescribed antibiotics within the past one month; ²⁾Subjects unvaccinated with PCV or Hib were defined as children who had never been vaccinated with PCV or Hib vaccine before.

³⁾ P=0.9666; ⁴⁾ P= 1; ⁵⁾ P=0.2733; ⁶⁾ P= 0.1747; * P $\leq$ 0.0001;

* We defined the bacteria isolated from more than 10% of children as main bacteria.