〈Brief Report〉

Microfloral analysis of pediatric respiratory syncytial virus infection, with and without the use of antibiotics

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Objectives To describe, in detail, the influence of antibiotics on the microflora of children with respiratory syncytial virus (RSV) infection.

Methods Eight hospitalized infants with RSV infection were included in the study. To examine the change in the microflora before and after the use of antibiotics, we simultaneously collected nasopharyngeal and stool swabs from each patient at 3 points; before antibiotics use, during antibiotics use, after use. The use of antibiotics was determined by clinicians. In 6 patients, antibiotics were used, and in 2, they were not. We analyzed the nasopharyngeal and fecal microflora through the clone library method, using amplified fragments of the 16S ribosomal RNA gene with universal primers.

Results With regards to nasopharyngeal microflora, of the 6 patients who were prescribed antibiotics, 4 had pathogenic bacteria including *Haemophilus influenzae* and *Moraxella catarrhalis*, on admission, and only 1 patient had *M. catarrhalis* 3–5 days after admission. However, in 3 patients, *M. catarrhalis* was the dominant bacterium, 1–2 weeks after hospital discharge. Among the patients who were not

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prescribed antibiotics, the representative pathogens of childhood pneumonia were not observed in any of the patients 1–2 weeks after hospital discharge, despite them having those pathogens on admission or 3–5 days after. With regards to fecal microflora, the number of bacterial species decreased after antibiotics use, except in 1 patient. One to 2 weeks after hospital discharge, the number of bacterial species was lower than that observed on admission, in all the cases in which antibiotics were used.

Conclusions The number of bacterial species both in the nasopharyngeal and fecal samples decreased after antibiotic use. The change in the dominant bacteria of the nasopharynx may be different between the cases in which antibiotics were administered and those in which they were not. These results suggest that clinicians should administer antibiotics, taking into consideration their influence on patients' microflora.

1. Introduction

Respiratory tract infections (RTIs) occur commonly in children. RTIs are induced by many microorganisms, and, in children, viruses are the major cause[1)]. The respiratory syncytial virus (RSV) is one of the major causative viruses of RTI in children[1)]. Generally, patients with RSV infection receive symptomatic treatment. If the patients are considered as having a bacterial co-infection, they are treated with antibiotics. However, it is not always easy to assess if patients have a bacterial co-infection, at the first medical examination. Moreover, the bacterial co-infection rates of RTIs, caused by RSV, vary, by study design[1,2)]. Therefore, in the clinical setting, antibiotics are sometimes prescribed to children with RSV infection, even when they are not required.

The use of antibiotics has its own demerits. Diarrhea is one of the common side effects of antibiotics use. Although several previously conducted studies have suggested that the use of antibiotics influences microflora, those reports used bacterial cultures[3–5)]. The technique involved in bacterial culture is relatively easy and can be conducted easily in hospitals. However, in bacterial cultures, non-culturable bacteria cannot be detected, and the percentage of each bacteria in a microflora cannot be estimated. To overcome these drawbacks, a method using a sequencer and polymerase chain reaction (PCR) for 16S ribosomal RNA was developed[6)]. To examine, in detail, the influence of antibiotics on the microflora of children with RSV infection, we analyzed nasopharyngeal and fecal microflora simultaneously through this method, before and after antibiotics were used. In this study, we focused on the relationships between viral infection, use of antibiotics and microflora. To the best of our knowledge, there are no reports, till date, on how antibiotics change both nasopharyngeal and stool microflora, using this new molecular biological method. We aimed to estimate the change in the microflora of children with viral infection, and, thereby, guide the proper use of antibiotics in these patients.

2. Materials and Methods

2.1. Participants

The patients in the present study were hospitalized infants with RSV infection, as diagnosed by rapid test kits (Chiba University: RSV examen™, Becton Dickinson and Company; Franklin Lakes, NJ. Chiba Rosai hospital and Chiba Children's hospital: QuickNavi™ RSV, DENKA SEIKEN Co., Ltd.: Tokyo, Japan), between September 2014 and March 2015, in Chiba University Hospital, Chiba Rosai Hospital and Chiba Children's Hospital. We excluded patients in whom antibiotics were administered within 2 weeks from the date of hospital admission. We also excluded patients in whom the injection of palivizumab, an anti-RSV monoclonal antibody, was recommended. Written informed consent was obtained from the participants' guardians.

2.2. Study protocol

To examine the change in the microflora, before and after the use of antibiotics, we collected nasopharyngeal and stool swabs, simultaneously, from each patient at 3 points; on admission (before antibiotic use), 3–5 days after admission (during antibiotic use), and 1–2 weeks after discharge from the hospital (after antibiotic use). The use of antibiotics was determined by clinicians. The severity of the condition, in each patient, was scored, as previously described[7,8]. The study protocols were approved by the Ethics Committee of the Chiba University (approval no. 1857), the Chiba Rosai hospital (approval no. 26–22), and the Chiba Children's hospital (approval no. 2014-01-02). This study was carried out in accordance with the protocol.

2.3. Analysis of the nasopharyngeal and fecal microflora

We analyzed the nasopharyngeal and fecal microflora through the clone library method, using amplified fragments of the 16S ribosomal RNA gene with universal primers[9–12]. After bacterial cell staining with ethidium bromide, we counted the total number of bacterial cells in the sample[13]. DNA was extracted from the samples by adding 2 ml of sterilized water and vigorously shaking the samples with sodium dodecyl sulfate (final concentration: 3.0%) and glass beads[9,11]. Then, the total number of bacterial cells in the sample was counted again, and we evaluated the efficacy of cell lysis, as previously described[9–12]. PCR of 16S rRNA gene was subsequently performed, using universal primers, under the following conditions; 95℃ for 5 minutes, 30 cycles at 94℃ for 1 minute, 50℃ for 1 minute, and 72℃ for 2 minutes, followed by a final extension at 72℃ for 7 minutes[11,13]. The PCR amplicons were cloned using a TOPO TA cloning kit (Invitrogen; Carlsbad, CA). Of them, a total of 96 colonies were randomly selected for the sequencing analysis. The analysis was performed using a 3130xl Genetic Analyzer (Applied Biosystems), according to the instrument manual. A Blast search was used to determine the species of each clone library. These analyses were performed as soon as possible, after the samples were collected.

3. Results

3.1. Patients' characteristics

A total of 8 patients were assessed in this study; of them, antibiotics were used in 6 (ampicillin; 1 patient, ampicillin/sulbactam; 5 patients). As shown in Table 1, the age of the patients in whom antibiotics were used was 8.3±5.2 months (mean±standard deviation, SD), while that of the patients in whom antibiotics were not used was 4.0 months. The SD of the latter could not be calculated because of the small number. All the patients had a lower respiratory infection, such as bronchitis, bronchiolitis, and pneumonia. With regards to the severity of the RTIs, in this study, 6 out of the 8 patients were categorized as having a mild form of the infection, while 1 each were considered as having moderate and severe infection, respectively.

3.2. Total bacterial cell numbers and cell lysis

All the samples collected in the present study had the sufficient number of bacterial cells for analysis. The medians of the total bacterial cell number are shown in Table 2. A larger number of bacterial cells was found in the stool than the nasopharynx. Furthermore, the total number of bacterial cells tended to decrease after admission, regardless of the use of antibiotics. The efficiency of cell lysis ranged from 74.5% to 99.9% (median 91.0%).

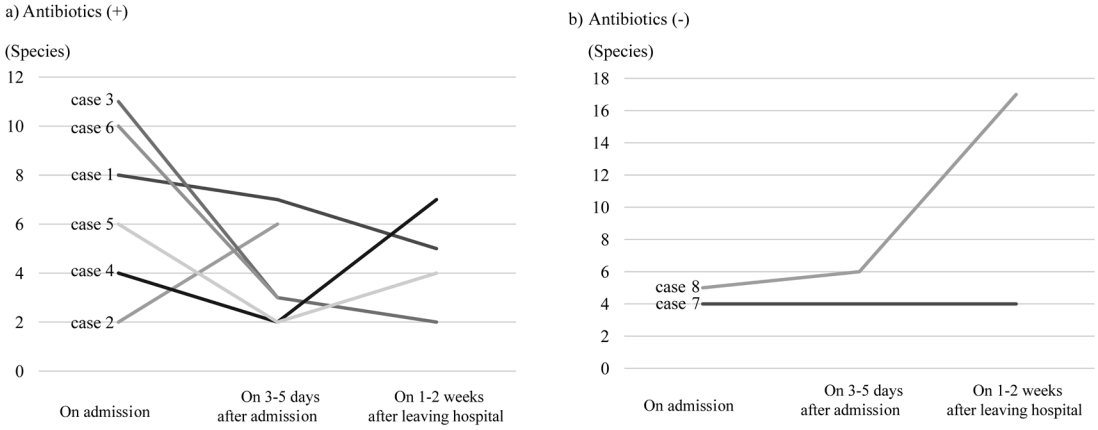
Table 1. Clinical features of the patients in the present study

	The cases of given Antibiotics	The cases of not given Antibiotics
Number of the patients	6	2
Age (months); mean ± SD	8.3 ± 5.2	4.0
Sex		
Male	4	1
Female	2	1
Clinical diagnosis		
bronchitis	0	1
bronchiolitis	2	1
pneumonia	4	0
Severity of disease		
mild	4	2
moderate	1	0
severe	1	0

Table 2. The median of total bacterial cell numbers

		on admission	on 3-5 days after admission	on 1-2 weeks after leaving hospital
Antibiotics (+)	Nasopharynx	6.2×10^6 cells/2ml	8.3×10^5 cells/2ml	6.2×10^6 cells/2ml
	Stool	1.4×10^8 cells/2ml	1.0×10^8 cells/2ml	3.4×10^7 cells/2ml
Antibiotics (-)	Nasopharynx	1.2×10^7 cells/2ml	3.1×10^6 cells/2ml	2.4×10^6 cells/2ml
	Stool	2.3×10^9 cells/2ml	1.3×10^8 cells/2ml	1.3×10^8 cells/2ml

Fig. 1. The number of nasopharyngeal bacterial species in a) the patients in whom antibiotics were used and b) patients in whom antibiotics were not used



3.3. Nasopharyngeal microflora

We analyzed 22 nasopharyngeal swabs, including 8 samples on admission, 8 samples 3–5 days after admission, and 6 samples 1–2 weeks after hospital discharge. In the case of 2 patients, samples could not be collected at the time of hospital discharge, as 1 patient was transferred to another hospital and the other did not allow the collection of nasopharyngeal samples 1–2 weeks after discharge.

The number of nasopharyngeal bacterial species is shown in Fig. 1. As shown in Fig. 1, the number tended to decrease after the use of antibiotics, while the number of patients in whom antibiotics were not used was either constant or tended to increase. Furthermore, we examined the change in the dominant bacteria, in each sample (Table 3). Of the 6 patients in whom antibiotics were prescribed, the representative pathogens of childhood pneumonia, including *Haemophilus influenzae* and *Moraxella catarrhalis*, were noted in 4 patients, on admission, while *M. catarrhalis* was observed in 1 patient, 3–5 days after admission. However, in 3 patients, *M. catarrhalis* was the dominant bacterium 1–2 weeks after hospital discharge. However, among patients who were not prescribed antibiotics, none of the patients had the representative pathogens of child-

Table 3. Dominant bacterium of each nasopharyngeal sample

	case	on admission (%*)	on 3-5days after admission (%*)	on 1-2 weeks after leaving hospital (%*)
	1	*Pasteurella pneumotropica* (60.9)	*Streptococcus pseudopneumoniae* (44.1)	***Moraxella catarrhalis***# (64.8)
	2	***Haemophilus influenzae***# (98.9)	***Moraxella catarrhalis***# (71.3)	no data
Antibiotics	3	***Moraxella catarrhalis***# (44.3)	*Streptococcus pseudopneumoniae* (76.1)	***Moraxella catarrhalis***# (98.9)
(+)	4	***Moraxella catarrhalis***# (79.3)	*Streptococcus pseudopneumoniae* (75.5)	*Streptococcus pseudopneumoniae* (56.3)
	5	***Moraxella catarrhalis***# (88.3)	*Streptococcus pseudopneumoniae* (98.9)	***Moraxella catarrhalis***# (96.7)
	6	*Haemophilus pittmaniae* (47.7)	*Simonsiella muelleri* (84.6)	no data
Antibiotics	7	*Staphylococcus aureus* (52.1)	***Streptococcus pneumoniae***# (95.1)	*Staphylococcus aureus* (83.2)
(-)	8	***Moraxella catarrhalis***# (46.4)	*Moraxella lincolnii* (45.1)	*Neisseria mucosa* (28.1)

* The percentage of the dominant bacteria among the all detected bacterium from each sample

Bacterium with bold letters were the representative pathogen of child pneumonia[2)].

hood pneumonia 1–2 weeks after hospital discharge, although they had those pathogens on admission or 3–5 days after admission.

3.4. Fecal microflora

Twenty-three stool samples, including 8 samples collected on admission, 8 samples 3–5 days after admission, and 7 samples 1–2 weeks after hospital discharge were analyzed. One sample could not be obtained due to hospital transfer. The number of bacterial species in each stool sample is shown in Fig. 2. The number decreased after the use of antibiotics, except in the case of one patient (case 3). However, the number decreased in case 3 too, after hospital discharge. One to 2 weeks after hospital discharge, the number of bacterial cells was lower than that observed on admission, in all the cases in which antibiotics were used. We could not find a tendency for the reduction in the number of bacterial cells and species in the no antibiotics admission cases. Furthermore, in the present study, we could not detect the dominant bacterium in the stool.

3.5. Correlations between nasopharyngeal and fecal microflora during the clinical course

Table 4 shows the number of bacterial species in the nasopharyngeal and fecal microflora, in the patients, during the clinical course. The number of bacterial species in both the nasopharynx and fecal microflora decreased in 4 of 6 patients treated with antibiotics. One to 2 weeks after antibiotic treatment, in 2 specimens—the nasopharynx specimen of case 4 and fecal specimen of case 6—were recovered number of bacterial species before antibiotic treatment.

Fig. 2. The number of fecal bacterial species in a) the patients in whom antibiotics were used and b) those in whom antibiotics were not used

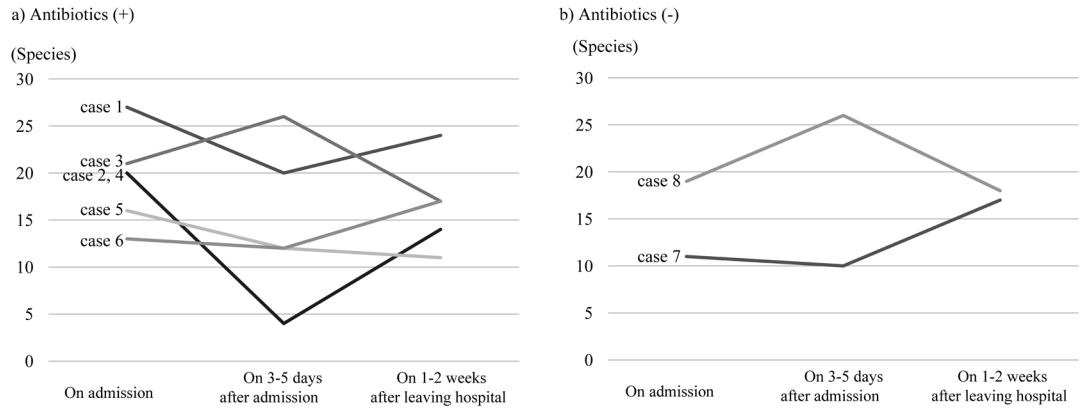


Table 4. Number of bacterial species of nasopharyngeal and fecal microflora in the patients during clinical course

Case number	Antibiotics	Specimen	Number of species on admission	Number of species on 3-5 days after admission	Number of species on 1-2 weeks after leaving hospital
Case 1	SBT/ABPC	Nasopharynx	8	7	5
		Stool	27	20	24
Case 2	ABPC	Nasopharynx	2	6	ND
		Stool	20	4	ND
Case 3	SBT/ABPC	Nasopharynx	11	3	2
		Stool	21	26	17
Case 4	SBT/ABPC	Nasopharynx	4	2	7
		Stool	20	4	14
Case 5	SBT/ABPC	Nasopharynx	6	2	4
		Stool	16	12	11
Case 6	SBT/ABPC	Nasopharynx	10	3	ND
		Stool	13	12	17
Case 7	Not given	Nasopharynx	4	4	4
		Stool	11	10	17
Case 8	Not given	Nasopharynx	5	6	17
		Stool	19	26	18

4. Discussion

In this study, we described the change in the microflora, in children with a viral infection. Our main findings are as follows: First, the number of bacterial species in both the nasopharyngeal and fecal samples decreased after the use of antibiotics. Second, the change in the dominant bacteria of the nasopharynx may be different between cases in which antibiotics were administered and those in which they were not. Reports suggest that the divergence of the microflora in the stool decreased through antibiotic administration[14,15]. Furthermore, Brook *et al* and Konno *et al* showed, through bacterial cultures, that antibiotics affected the nasopharyngeal microflora[4,5]. Those studies, however, did not examine the microflora of the upper respiratory tract, using molecular biological methods. In contrast, we examined both nasopharyngeal and fecal microflora, simultaneously, using the clone library method. As a result, we could accurately evaluate fluctuations in the microflora, in the nasopharynx and stool, including bacteria that are hard to culture.

In the present study, we focused on patients with a viral infection. Hamano-Hasegawa *et al.* reported that 15.2% of pediatric patients with community-acquired pneumonia have bacterial co-infection, using the broad-range PCR method[1]. Hishiki *et al.* showed that 45% of patients with lower respiratory tract infection, caused by RSV—which is one of the most common causes of infection in children—have bacterial co-infection, through the washed sputum culture method[2]. In our study too, 5 of 8 patients had the pathogenic bacteria of pediatric pneumonia on admission, and another patient had *Streptococcus pneumoniae* 3–5 days after admission. However, in the patients in whom antibiotics were not prescribed, those pathogenic bacteria were not detected 1–2 weeks after hospital discharge. Based on this result, it is suggested that not all detected pathogenic bacteria may be causative pathogens in patients with a viral infection; in fact, bacterial infections may be more over-diagnosed than expected.

In this study, the number of bacterial species decreased after the use of antibiotics, in the nasopharyngeal swab samples and stool samples, while the number of bacterial species did not decrease, in cases in which antibiotics were not administered. Many studies reported that the divergence of fecal microbiota decreased through the use of antibiotics[14–17]. The divergence of nasopharynx microbiota may also decrease through antibiotics use, with regular dosage.

The dominant bacterium of the nasopharynx differed, depending on the phase at which each sample was collected. Three to 5 days after admission, in the cases in which antibiotics were administered, the changes in the number of bacterial species could be attributed to the antibacterial activity of the antibiotics. After the use of antibiotics, pathogenic bacteria, including *H. influenzae* and *M. catarrhalis*, disappeared. However, *M. catarrhalis* was recovered 1–2 weeks after hospital discharge, in cases in which antibiotics were used. Brook *et al* suggested that potential pathogens including *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* rebound faster in those treated with amoxicillin/clavulanate compared to those in whom cefdinir was administered, although the

study used the bacterial culture method[5]. We used the clone library method, and, therefore, were able to evaluate a large number of bacteria, including those species that are difficult to culture. Our results were compatible with those of the study by Brook *et al.*[5].

To estimate the pathogens in patients with RTI, at the initial diagnosis, may be difficult as the symptoms of RTI do not vary from those of other microorganisms. In addition, the results of bacterial culture, which is the standard for bacterial infection, are obtained only after a few days. A substantial number of instances of bacterial and viral co-infections have been reported[1,2]. Therefore, it can be assumed that antibiotics are used in several cases even when they are not required. Some reports suggest that the use of antibiotics in infants may raise the risk of asthma[18,19]. It was thought that these demerits were caused by the change in the microflora[18]. Our results showed that antibiotics may change the microflora of both the nasopharynx and bowel.

Our study had some limitations. First, the number of patients in the present study was slightly small. Second, samples from two patients that could not be collected at all 3 points were also included in the present study. To confirm these effects of antibiotics on microflora, further studies are needed. Based on the results of the present study, clinicians are urged to use antibiotics taking into consideration their influence on microflora.

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Competing interests

The authors declare that they have no competing interests.

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