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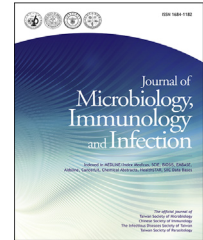

Short Communication

The microbiological characteristics of lower respiratory tract infection in patients with neuromuscular disorders: An investigation based on a multiplex polymerase chain reaction to detect viruses and a clone library analysis of the bacterial 16S rRNA gene sequence in sputum samples

Masato Ogawa [a,1], Takayuki Hoshina [a,*,1], Kaoru Haro [a], Tokiko Kumadaki [a], Masahiro Ishii [a], Yoshihisa Fujino [b], Kazumasa Fukuda [c], Koichi Kusuhara [a]

[a] *Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Japan*
[b] *Department of Preventive Medicine and Community Health, School of Medicine, University of Occupational and Environmental Health, Japan*
[c] *Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Japan*

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Abstract We performed gene amplification methods for the detections of bacteria and viruses using sputum samples to clarify the microbiological characteristics of lower respiratory tract infection in patients with neuromuscular disorders. The tendencies of higher proportion of respiratory virus detection and lower diversity of bacteria in sputum were observed.

* Corresponding author. Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. Fax: +81 93 691 9338.
E-mail address: hoshina@med.uoeh-u.ac.jp (T. Hoshina).
[1] Equally contributed authors.

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Respiratory viruses;
16S rRNA gene;
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Introduction

Neuromuscular disorders (NMD) have a high risk of developing severe lower respiratory tract infections (LRTIs),[1,2] because these patients generally have obstructive and restrictive respiratory disorders, which makes it difficult to clear sputum from the airways.[3] LRTIs in NMD patients are thought to be caused by various drug-resistant bacteria, including *Pseudomonas aeruginosa*,[4] colonizing in the respiratory tract. In contrast, we previously reported that antimicrobial agents without activity against *P. aeruginosa* were effective in the treatment of LRTI in these patients.[5] It is unclear whether these bacteria are the main cause of LRTIs in NMD patients. Furthermore, the contribution of viruses to the development of LRTIs in these patients also remained uncertain.

In the present study, in addition to the bacterial culture, we performed a multiplex polymerase chain reaction (PCR) to detect viruses and a clone library analysis of the bacterial 16S rRNA gene sequence using sputum samples to clarify the microbiological characteristics of LRTIs in NMD patients.

Materials and methods

Study population

This prospective study included NMD patients with a permanent tracheostomy, who received regular care from pediatricians in our hospital and were hospitalized for LRTI from April 1, 2014, to April 30, 2017. All patients were bedridden and had an IQ or DQ of $\leq$20. The clinical information of the patients were collected using a standardized case report form. LRTIs were diagnosed when they showed a worsening of cough and sputum production, accompanied by ausculatory findings of abnormal breath sounds.[10] Informed consent was obtained from the patient's parents in all cases. Our study was approved by the Institutional Review Board of our institution.

Samples collection

Sputum samples were obtained by inserting a collection tube into the patient's trachea in asymptomatic condition preceding the development of the LRTI (non-infectious state) and on the development of LRTI (infectious state). All but one patient did not take antimicrobial agents more than one month before non-infectious state and between non-infectious and infectious states. One patient (No. 1-1) took one dose of oral antimicrobial agent immediately before sampling in infectious state (Supplementary Table 1). Part of each sample was processed for Gram staining and bacterial culturing routinely performed in the bacteriology laboratory of our hospital. The remainder of the samples was stored at -20 °C for the detection of viruses and the bacterial gene analysis.

The detection of representative respiratory viruses

Viral RNA was extracted using Ribo_spin vRD (GeneAll, Seuol, Korea) in accordance with the manufacturer's instructions. The isolated RNA was amplified using the one-step RT-PCR of Seeplex RV15 OneStep ACE detection (Seegene Inc., Seoul, Korea) which can detect the following 15 respiratory viruses; parainfluenza virus 1/2/3/4, adenovirus, coronavirus 229E/NL63/OC43, rhinovirus A/B/C, influenza virus A and B, respiratory syncytial virus A and B, bocavirus 1/2/3/4, human metapneumovirus, and enterovirus. The methods of PCR amplification were described in supplementary methods.

Total bacterial cell counts and cell lysis efficiency analysis

The methods for calculating total bacterial cell counts were described in supplementary methods. The cell lysis efficiency was calculated by use of the following expression: [100 $-$ (post-extraction number/pre-extraction number) $\times$ 100]. To eliminate the bias due to the bacteriolysis rate, we made an effort to sustain more than 80% of the cell lysis efficiency.

Bacterial analysis in sputum using clone library analysis

We performed a clone library analysis of the bacterial 16S rRNA gene sequence using sputum samples. The methods of DNA extraction, PCR amplification of the 16S rRNA gene, clone library construction, determination of nucleotide sequences and homology searching were described in supplementary methods.

Statistical analysis

The paired *t*-test or Wilcoxon signed-rank test was performed using the SPSS II software program (version 11.0 J for Windows; SPSS Inc., Chicago, IL and IBM, Armonk, NY) to compare the differences between the quantitative values. Matched case–control studies were performed using the Stata software program (version 15; Stata Corporation, College Station, TX) to compare the proportions of virus detection in the infectious and non-infectious states. *P*-values of $\leq$0.05 were considered to indicate statistical

significance. Odds ratios (ORs) and 95% confidence intervals (CIs) were also shown in the virological analysis. The Simpson index was calculated to compare the bacterial diversity of sputum. The method for the calculation was described in supplementary methods. The index values ranged from 0.0 to 1.0. A value of close to 1.0 indicates that a sample has higher diversity.

Results

During the investigation period, sputum samples from both infectious and preceding non-infectious states were obtained in 15 episodes from 7 patients. The median sampling interval between the non-infectious and infectious states was 7 weeks (2—64 weeks). The characteristics of the patients on the development of LRTI are shown in Supplementary Table 1. Antimicrobial agents were administrated to all patients based on the suspicion of bacterial infection. All patients improved after the administration of the appropriate treatments.

Viruses were only detected from 3 non-infectious samples (20%), and were detected in12 infectious samples (80%) (Table 1); the difference in the proportion of detection in the infectious and non-infectious state was statistically significant (OR, 9.00; 95% CI, 1.14—71.03; $P = 0.014$).

The bacterial cell counts in sputum samples obtained in infectious (median, 1.8×10^7 cells/mL; range, 8.0×10^5—6.0×10^8) and non-infectious (median, 2.4×10^7 cells/mL; range, 1.4×10^5—6.8×10^7) states did not differ to a statistically significant extent (Supplementary Fig. 1). The Simpson index values of samples obtained in the infectious state were lower than those of the samples obtained in the non-infectious state in 11 of the 15 episodes (73.3%), indicating that the bacteria in sputum tended to show less diversity on the development of LRTI (Table 2). In both the infectious and non-infectious states, the top three bacteria detected by the clone library analysis were not always identified by culturing. The proportion of concordance between the two methods was 66.7% and 60.0% in the infectious and non-infectious states, respectively (Supplementary Table 2). In particular, *P. aeruginosa* was detected in most of the culture, whereas it was rarely detected as top three bacteria in the clone library analysis. In most cases, the predominant bacterium detected by the clone library analysis in the samples obtained in the infectious state was not consistent with the predominant bacterium detected in the non-infectious state (Supplementary Table 2). However, when *Haemophilus influenzae* was detected as the predominant bacterium in the non-infectious samples, the same bacterium tended to be predominant in the samples obtained in the subsequent infectious state (4 of 5 episodes) (Supplementary Table 2).

Table 1 Comparison of identified viruses by episode between infectious and non-infectious states.

Case and episode	Infectious state	Non-infectious state
1-1	N.D.	N.D.
2-1	Rhinovirus	Rhinovirus
3-1	Rhinovirus	N.D.
3-2	Rhinovirus	N.D.
4-1	Rhinovirus	N.D.
4-2	Rhinovirus	N.D.
5-1	Rhinovirus	N.D.
5-2	Parainfluenza virus 1, 2, 3	Parainfluenza virus 1, 2, 3 Coronavirus 229E/NL63
5-3	N.D.	Rhinovirus
5-4	Coronavirus 229E/ NL63 Rhinovirus	N.D.
5-5	N.D.	N.D.
6-1	Parainfluenza virus 4 Rhinovirus	Parainfluenza virus 4
6-2	Enterovirus	N.D.
7-1	Rhinovirus	N.D.
7-2	Parainfluenza virus 4	N.D.

A multiplex polymerase chain reaction kit was used for the detections for parainfluenza virus 1/2/3/4, adenovirus, coronavirus 229E/NL63/OC43, rhinovirus A/B/C, influenza virus A and B, respiratory syncytial virus A and B, bocavirus 1/2/3/4, human metapneumovirus and enterovirus. N.D.: not detected.

Discussion

Mixed viral and bacterial infections have been reported in cases of community-acquired pneumonia.[6,7] The presence of viruses in human tracheal epithelium cells increases their susceptibility to bacterial infection.[8] Moreover, the virus also impairs the immune responses to bacterial products in human alveolar macrophages.[9] Our study

Table 2 Comparison of Simpson index by episode between the development of LRTI (infectious state) and asymptomatic state (non-infectious state).

Case and episode	Infectious state	Non-infectious state[a]
1-1	0.3634	0.8516
2-1	0.0222	0.3023
3-1	0.8793	0.8337
3-2	0.8911	0.2274
4-1	0.5948	0.7275
4-2	0.4897	0.5675
5-1	0.6107	0.7737
5-2	0.4835	0.5861
5-3	0.8846	0.6573
5-4	0.2716	0.5124
5-5	0.7821	0.9283
6-1	0.8421	0.5244
6-2	0.8018	0.8801
7-1	0.8921	0.8977
7-2	0.7639	0.8639

[a] This indicates that patients are asymptomatic, and do not take any antimicrobial agents more than one month before sampling.
All patients did not take any antimicrobial agents between non-infectious and infectious states. LRTI: lower respiratory tract infection.

suggested that the primary viral LRTI might trigger bacterial infection because of the frequent detection of respiratory viruses in the development of bacterial LRTI.

In this study, the bacterial load in NMD patients was high even in their asymptomatic condition, indicating that it might not be a useful marker of bacterial LRTI. Furthermore, bacteria that were difficult to detect routine bacteriological culturing methods were occasionally detected from these patients. Thus, gene amplification methods may be superior to culturing methods for the diagnosis of LRTIs in these patients. In fact, the clone library analysis indicated that the detection of low bacterial diversity in sputum might be useful for the diagnosis of bacterial LRTIs in NMD patients. In the cases with the discordance between the result of the clone library analysis and the culture, oral bacteria or bacteria that were less culturable were mainly detected as top three bacteria in the clone library analysis. It is also speculated that oral bacteria are assumed to be indigenous and tend to be ignored in sputum samples in ordinary clinical settings. In addition, *P. aeruginosa* was detected from most sputum cultures, while it was rarely detected in the clone library analysis. Although the data were not shown, the clinical symptoms in most of the eligible patients improved following the administration of antimicrobial agents without activity against *P. aeruginosa*, suggesting that the frequency of the bacterium as a causative pathogen of LRTI in NMD patients might not be as high as previously thought. Furthermore, although the sample size was small, the clone library analysis suggested that it may be useful to select an antimicrobial agent targeting the bacterium on the development of LRTI, when *H. influenzae* is detected in asymptomatic patients.

The clone library analysis method has more technical limitations than the metagenome sequencing analysis. On the other hand, this method has beneficial effects in that it can exactly identify bacteria at the species level because of the relatively longer length of sequences compared to the next-generation sequencing. We performed the clone library analysis in this study because it was most important to identify the causative bacteria at the spices level. However, in the future, the metagenome sequencing analysis may be superior to clone library analysis in the identification of bacteria by the further improvement of the technology.

This study has some limitations. First, it was impossible to completely amplify all of the bacterial 16S rRNA genes. Although the sensitivity of the primers for the bacterial species used in this study was approximately 92%, all pathogenic bacteria could be detected by using these primers. Second, there was no method to evaluate whether the bacteria that were predominantly identified by the clone library analysis were the real causative pathogens of LRTI. In general, an increase in the bacterial load in the sputum is associated with the development of LRTIs.[7,10] Our hypothesis that predominant bacteria would become causative pathogens may be justified. Finally, study population was relatively small; this could have affected the accuracy of the statistical analysis.

In conclusion, respiratory viruses were detected at higher proportions and the diversity of bacteria in sputum tended to be lower on the development of LRTIs in NMD patients. Further large-scale studies using this method should be performed to investigate the etiology and indicate appropriate antimicrobial therapies for NMD patients with LRTIs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2019.01.002.