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The optimal duration of antimicrobial therapy for lower respiratory tract infection in patients with neuromuscular disorders based on a clone library analysis of the bacterial 16S rRNA gene sequence

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ABSTRACT

Objectives: The aim of this study is to determine the optimal duration of antimicrobial therapy for lower respiratory tract infection (LRTI) in neuromuscular disorder (NMD) patients.
Methods: This prospective study included 13 episodes from 9 NMD patients hospitalized for bacterial LRTI. Sputum samples were collected from these patients during the three consecutive days after their admission. Bacterial cell counts and the proportion of the most predominant bacterium identified by a clone library analysis of the bacterial 16S rRNA gene sequence in the samples obtained before antimicrobial therapy were serially investigated.
Results: All episodes were initially treated with ampicillin/sulbactam. In 12 episodes with a therapeutic effect, the bacterial cell counts in the samples obtained on the third day of therapy were significantly lower than those before therapy ($P = 0.0013$). In most of these episodes, the most predominant bacterium in the sample obtained before therapy was undetectable by the third day of therapy. In the one patient without a therapeutic effect, neither the bacterial cell counts nor the proportion of the most predominant bacterium in the sample obtained before therapy decrease after therapy.
Conclusion: Short-term antimicrobial therapy is sufficiently effective for LRTI in NMD patients if the initial therapy is effective.

Introduction

Lower respiratory tract infection (LRTI) is one of the most common diseases in children and is often caused by bacteria (McIntosh, 2002). Although the introduction of pneumococcal conjugate vaccine has reduced the number of pediatric patients with bacterial LRTI (Griffin et al., 2013; Haro et al., 2017; Naito et al., 2016), many patients who need to be treated with intravenous antimicrobial agents are still hospitalized for the disease. For the appropriate antimicrobial treatment of bacterial LRTI, it is important to determine the optimal duration of the therapy as well as to choose appropriate antimicrobial agents. Guidelines for the treatment of pediatric LRTI in developed countries, including Japan, suggest that 5–10 days of antimicrobial therapy is ideal for previously-healthy patients with bacterial pneumonia (Bradley et al., 2011; Harris et al., 2011; Mikasa et al., 2016).

Neuromuscular disorder (NMD) carries a high risk of developing LRTIs, as patients with this disorder generally have obstructive and restrictive respiratory disorders, which make it difficult to clear sputum from the airways (Millman et al., 2016; Wilkesmann et al., 2007). LRTIs in NMD patients had been thought to be caused by drug-resistant bacteria, including *Pseudomonas aeruginosa*, that colonize in the respiratory tract (Thorburn et al., 2009). However, our previous study using a clone library analysis of the bacterial 16S rRNA gene sequence indicated that the frequency of LRTI caused by these bacteria was not as high as previously thought, and

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that the clinical symptoms in most NMD patients improved following the administration of antimicrobial agents without activity against *P. aeruginosa* (Ogawa et al., 2019). Despite these promising findings regarding which agents are most effective, the optimal duration of antimicrobial therapy for LRTI in NMD patients remains unclear.

To determine the optimal duration of antimicrobial therapy for LRTI in NMD patients, we investigated the serial changes in the microbiome of sputum samples obtained from NMD patients with bacterial LRTI using a clone library analysis.

Materials and methods

Study population

This prospective study included NMD patients with a permanent tracheostomy, who were admitted to the Department of Pediatrics at the Hospital of University of Occupational and Environmental Health, Japan from March 1, 2016 to April 30, 2017, under suspicion of bacterial LRTI. All patients were bedridden and had an Intelligence Quotient (IQ) or Developmental Quotient (DQ) of $\leq$20. The clinical information of the patients was collected using standardized case report form.

A LRTI was diagnosed when the patients had a cough and sputum production as clinical symptoms, accompanied by abnormalities on chest auscultation (Gadsby et al., 2016). A bacterial etiology of LRTI were suspected when a patient showed an increased white blood cell counts ($>15 \times 10^9$/L) and/or elevated serum C-reactive protein (CRP) levels (>20 mg/L). In patients with leukocytosis and/or elevated serum CRP levels, bacterial LRTI was diagnosed when phagocytized bacterial cells were seen on the Gram stain smear of the sputum sample. The presence of consolidation on chest X-ray was assessed by two of the authors (T.H., K.K.). Informed consent was obtained from all patients' parents. Our study was approved by the Institutional Review Board of the University of Occupational and Environmental Health, Japan.

Policy of antimicrobial therapy for NMD patients hospitalized for bacterial LRTI

In our hospital, ampicillin/sulbactam (120–150 mg/kg/day, maximal dose: 6 g/day) was initially administrated intravenously for NMD patients because *Moraxella catarrhalis* was major causative pathogen of LRTI in these patients (Hoshina et al., 2010). If improvement in the clinical symptoms was achieved promptly, the initial therapy was discontinued after roughly 5 days. If no therapeutic effect of the initial antimicrobial agent was shown after 2–3 days, we re-evaluated the diagnosis of the patient and investigated the results of microbiological examinations. Changes to the treatment were considered after the re-evaluation and further investigation.

Sample collection

Sputum samples were collected from NMD patients with LRTI during the three consecutive days after admission. The samples were obtained by inserting a collection tube into patient's trachea. Part of each sample was processed for Gram staining and bacterial culturing. These procedures were routinely performed in the bacteriology laboratory of our hospital. The remainder of the samples was stored at −20 °C for the bacterial gene analysis.

Total bacterial cell counts and cell lysis efficiency analysis

Each sputum sample (100 μL) was added to 900 μL of ethidium bromide solution [100 μg/mL in 0.1 M phosphate buffer (pH 8.5), 5% NaCl, 0.5 mM ethylenediaminetetraacetic acid-2Na] and was left for 10 min at room temperature. After filtering the mixture through a 0.2-μm-pore filter (Millipore, Bedford, MA), we counted bacteria on the filter in randomly chosen 30 fields using a microscope. The average number of bacteria per milliliter was calculated. The number of the remaining bacteria after the DNA extraction was also calculated by the same method to check the cell lysis efficiency. The efficiency was calculated by use of the following expression; [100 - (post-extraction number/pre-extraction number) x 100]. To eliminate the bias due to the bacteriolysis rate, we made an effort to sustain the cell lysis efficiency of >80%.

DNA extraction

DNA was extracted from sputum samples by vigorous shaking together with sodium dodecyl sulfate solution (final concentration, 3.0%) and glass beads as previously reported (Ogawa et al., 2019).

PCR amplification of the 16S rRNA gene using universal primers

The 16S rRNA gene was amplified using a Veriti thermocycler (Applied Biosystems, Foster City, CA, USA). Reaction mixtures containing the universal primer set (E341F: 5'-CCTACGGGAGGCAGCAG-3' and E907R: 5'-CCGTCAATTCMTTTRAGTTT-3') and AmpliTaq Gold DNA polymerase LD (Applied Biosystems) were incubated in a thermocycler at 96 °C for 5 min, followed by 30 cycles at 96 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min and then one cycle for the final elongation step at 72 °C for 7 min.

Clone library construction and determination of nucleotide sequences

The PCR products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 96 colonies were randomly selected from each clone library for sequencing analysis. The partial fragments of the cloning vectors (pCR 4) containing inserted PCR products were amplified with AmpliTaq 360 Master Mix, GC Enhancer and Pre-Seq primer set (F; 5'-GTTTTCCCAGTCACGACG-3' and R; 5'-CAGGGAACAGCTATGAC-3', Applied Biosystems). After the primers and deoxyribonucleotided triphosphate were eliminated from PCR mixture with an Exonuclease I and Alkaline Phosphatase (Shrimp) (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions, a 1 μL aliquot was used as a template for the sequencing reaction. The sequencing reactions were accomplished with primers M13 F and BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The nucleic acid sequences were determined on a 3130xl Genetic Analyzer (Applied Biosystems).

Homology searching

DNASIS Pro software (Hitachi Software Engineering, Tokyo, Japan) was used to check the quality and trimming of the sequences. Highly accurate sequences selected by KB basecaller v1.2 (Applied Biosystems) were compared with the 16S rRNA gene sequences of the type strains using the basic local alignment search tool algorithm. A phylotype sharing 97% or higher homology with the sequence of the type strain using the basic local alignment search tool algorithm was considered to be a presumptive species in this study.

The detection of representative respiratory viruses

Viral DNA and RNA were extracted using Ribo_spin vRD (GeneAll, Seuol, Korea) in accordance with the manufacturer's instructions. The isolated DNA and RNA were 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 PCR amplification was performed as previously described (Ogawa et al., 2019). Agarose gel electrophoresis with ethidium bromide staining was used to determine the presence of PCR products.

Statistical analysis

IBM SPSS statistics software program version 21 (IBM, Armonk, NY, USA) was used for the analysis. The Wilcoxon signed-rank test was used to compare the differences between the quantitative values in the bacteriological analysis. *P*-values <0.05 were considered to be statistically significant.

Results

Characteristics of the NMD patients

During the investigation period, sputum samples were obtained in 13 episodes from 9 NMD patients. The patients' characteristics are shown in Table 1. In just one episode (No. 8-3), ampicillin/clavulanate was given orally for 1 day before admission. After admission, ampicillin/sulbactam was initially administrated to all patients. All episodes but one improved after starting antimicrobial therapy. In one episode (No. 4-1), ampicillin/sulbactam was changed to piperacillin/tazobactam because of no therapeutic effect.

Presumptive causative pathogens based on the clone library analysis

DNA was extracted from sputum samples to perform the clone library analysis of the bacterial 16S rRNA gene sequence. The cell lysis efficiency was ≥80% in all samples (data not shown). The top three bacteria detected by the method were not always identified by culturing. The proportion of concordance between the two methods was 69.2% (Table 2). Gene amplification methods are often superior to culturing methods for the diagnosis of LRTIs in patients with chronic respiratory inflammation (Naito et al., 2017; Ogawa et al., 2019). Thus, we considered the most predominant bacterium detected by the clone library analysis to be the presumptive causative pathogens of LRTI. *Haemophilus influenzae* and *Pseudomonas aeruginosa* were the most predominant bacteria in five (38.4%) and three (23.1%) samples, respectively (Table2). Oral bacterium was the most predominantly detected pathogen in the remaining five samples.

Serial changes in the bacterial cell counts and proportion of presumptive causative pathogen after starting antimicrobial therapy

The numbers of bacteria stained by ethidium bromide were counted in each sputum sample (before DNA extraction was performed). In patients who showed a therapeutic effect, the total bacterial cell counts gradually decreased after starting the initial antimicrobial therapy (Figure 1). These counts on the third day of therapy (median, $10^{7.33}$ cells/mL; range, $10^{5.6}$ -$10^{9.4}$ cells/mL) were significantly lower than those before therapy (median, $10^{8.08}$ cells/mL; range, $10^{7.69}$ -$10^{8.97}$ cells/mL, $P = 0.0013$). In most patients who showed a therapeutic effect, the proportion of the most predominant bacterium detected by the clone library analysis in sputum samples obtained before therapy also decreased after therapy, and these bacteria were almost undetectable by the third day of treatment (Table 3).

In two of the episodes that showed a therapeutic effect, the total bacterial cell counts did not decrease after therapy. In one of these episodes (No. 8-3), the proportion of the most predominant bacterium detected in sputum samples obtained before therapy decreased after therapy (Table 3). In the other episode (No. 1-1), neither the total bacterial cell counts nor the proportion of the most predominant bacterium detected in the sputum samples obtained before therapy decreased after therapy. In one episode (No. 3-1), the total bacterial cell counts decreased after therapy, but the proportion of the most predominant bacterium detected in the sputum samples obtained before therapy increased after therapy (Table 3).

In one patient who was refractory to the initial antimicrobial therapy (ampicillin–sulbactam) (No. 4-1, Table 1) who was refractory to the initial antimicrobial therapy (ampicillin/sulbactam), the total bacterial cell counts ($10^{8.18}$ cells/mL) on the third day of the therapy were higher than those before therapy (10^{7} cells/mL), and the proportion of the most predominant bacterium (*H. influenzae*) in sputum samples obtained before therapy did not decrease after the initial antimicrobial

Table 1
Demographic and clinical characteristics of the eligible patients.

Case No.	Sex	Underlying diseases	Episode No.	Age of the onset (years)	WBC (10^9/L)	CRP (mg/L)	Diagnosis	Antimicrobial therapy	Duration of therapy (day)
1	F	Hydrocephalus due to brain tumor	1	2.5	13.7	67.3	Pneumonia	ABPC/SBT	6
			2	3.1	15.7	88.3	Bronchitis	ABPC/SBT	7
2	F	Cerebral palsy	1	7.5	11.3	85.5	Pneumonia	ABPC/SBT	5
3	M	Sequelae of hypoxic encephalopathy Chiari malformation	1	6.7	9.6	62.4	Pneumonia	ABPC/SBT	6
4	F	Cerebral palsy	1[a]	7.9	15.9	151.9	Pneumonia	ABPC/SBT → PIPC/TAZ	10
			2[b]	8.2	16.9	145.9	Pneumonia	ABPC/SBT	9
5	F	Cerebral palsy	1	10.4	8.5	21.1	Bronchitis	ABPC/SBT	6
6	M	Sequelae of hypoxic encephalopathy	1	11.5	22.8	54.1	Pneumonia	ABPC/SBT	8
7	M	Adenoleukodystrophy	1	16.8	11.6	104.4	Bronchitis	ABPC/SBT	5
8	M	Cerebral palsy	1[c]	16.2	7.2	91.7	Bronchitis	ABPC/SBT	6
			2	16.5	16.6	152.9	Bronchitis	ABPC/SBT	6
			3	17.5	8.9	26.8	Bronchitis	ABPC/SBT	7
9	F	Cerebral palsy	1	21.2	23.1	248.0	Pneumonia	ABPC/SBT	6

WBC: white blood cell, CRP: C-reactive protein, ABPC/SBT: ampicillin/sulbactam, PIPC/TAZ: piperacillin/tazobactam.
[a] Clinical symptoms and laboratory data improve not following four days initial treatment (ABPC/SBT) but after the change of antimicrobial therapy (PIPC/TAZ). PIPC/TAZ had been administrated for six days.
[b] Influenza virus was also isolated at the acute phase of the disease.
[c] Ampicillin/clavulanate was given orally for one day before admission.

Table 2
Comparison of identified bacteria between a clone library analysis and the conventional culturing method in the development of lower respiratory tract infection in patients with neuromuscular disorders.

Case and episode	Clone library analysis		Identified bacteria in cultivation	Gram stain[b]
	Predominant bacteria[a]	% of clones		
1–1	*Pseudomonas aeruginosa Porphyromonas catoniae Streptococcus parasanguinis*	31.0 19.5 16.1	*Pseudomonas aeruginosa Staphylococcus aureus* (MRSA)	GPC, GNR
1–2	*Mannheimia granulomatis Fusobacterium canifelinum Pseudomonas aeruginosa*	28.6 23.1 20.9	*Pseudomonas aeruginosa Serratia marcescens*	GPR, GNR
2–1	*Pseudomonas aeruginosa Micromonas micos*	98.6 1.4	*Pseudomona aeruginosa*	GNR
3–1	*Pseudomonas aeruginosa Fusobacterium canifelinum Streptococcus difficilis*	14.1 10.9 10.9	*Pseudomonas aeruginosa*	GPC, GPR, GNR
4–1[c]	*Haemophilus influenza Corynebacterium striatum Staphylococcus epidermidis*	82.6 4.7 3.5	*Haemophilus influenzae* (BLPACR) *Streptococcus pneumoniae* (PRSP) *Pseudomonas aeruginosa*	GNR
4–2	*Moraxella catarrhalis Staphylococcus aureus Haemophilus influenzae*	40.9 39.8 4.5	*Staphylococcus aureus* (MSSA) *Pseudomonas aeruginosa*	GPR
5–1	*Streptococcus oralis Nisseria perflava Fsobacterium canifelinum*	27.7 12.3 6.2	*Pseudomonas aeruginosa Stenotrophomonas maltophilia*	GPC, GNR
6–1	*Haemophilus influenza Corynebacterium tuberculostearicum*	98.9 1.1	*Haemophilus influenzae*	GNR
7–1	*Neisseria cinerea Pseudomonas aeruginosa Aggregatibacter aphrophilus*	37.8 17.8 15.6	*Pseudomonas aeruginosa Stenotrophomonas maltophilia*	GPR, GNR
8–1	*Haemophilus influenza Fusobacterium nucleatum Streptobacillus moniliformis*	73.6 12.7 6.9	*Pseudomonas aeruginosa Serratia marcescens Haemophilus influenzae* (BLNAS)	GNR
8–2	*Haemophilus influenza Fusobacterium nucleatum Streptobacillus moniliformis*	57.9 10.5 9.2	*Acinetobacter baumannii Pseudomonas aeruginosa*	GNR
8–3	*Haemophilus influenza Streptococcus oralis Corynebacterium striatum*	58.2 12.1 6.6	*Pseudomonas aeruginosa Stenotrophomonas maltophilia*	GNR
9–1	*Streptococcus oralis Corynebacterium striatum Streptobacillus moniliformis*	22.1 19.6 9.3	*Pseudomonas aeruginosa*	GPR

MRSA: methicillin-resistant *Staphylococcus aureus*, BLPACR: β-lactamase producing ampicillin-clavulanate resistant.
PRSP: penicillin-resistant *Streptococcus pneumoniae*, MSSA: methicillin-susceptible *Staphylococcus aureus*.
BLNAS: β-lactamase negative ampicillin susceptible, GPC: gram positive cocci, GPR: gram positive rods, GNR: gram negative rods.
[a] The data showed top three bacteria.
[b] The results show the bacterium with the largest number in bacteria detected by gram stain.
[c] Clinical symptoms and laboratory data improved not following initial treatment but after the change of antimicrobial therapy.

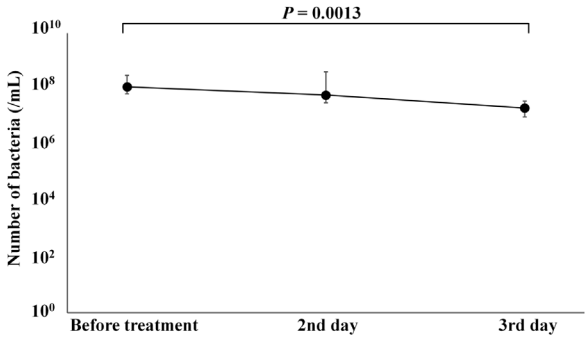

Figure 1. Serial changes of the total bacterial cell counts in sputum samples obtained from patients who showed a therapeutic effect by initial antimicrobial therapy (n=12). The numbers of bacteria stained by ethidium bromide were counted in part of each sputum sample (before DNA extraction was performed). The bottom and the top of the line correspond to 25th and 75th percentile points, respectively. The dot within the line represents median.

therapy (Figure 2). Both total bacterial cell counts and the proportion of the bacterium immediately decreased after switching to a second antimicrobial agent (piperacillin/tazobactam). Beta-lactamase-producing ampicillin-clavulanate-resistant *H. influenzae* was the presumptive causative pathogen of LRTI in this patient (Table 2).

The detection of respiratory viruses

In four of 13 episodes, respiratory viruses were detected from the sputum samples (rhinovirus and parainfluenza virus at No. 1-1, rhinovirus at No. 3-1 and No. 4-1 and enterovirus at No. 4-2). In 12 episodes, excluding 1 patient who was refractory to the initial antimicrobial therapy (No. 4-1), there were no marked differences in the total bacterial cell counts between 3 episodes with virus detection and 9 episodes without virus detection. In addition, no deflection of the detection of bacteria by a clone library analysis was seen in 4 episodes with virus detection. In our previous study, the proportion of NMD patients whose sputum samples were positive for respiratory viruses during the acute phase of LRTI was high (Ogawa et al. 2019). Thus, co-infection of bacteria and viruses was speculated to have been caused in these 4 episodes.

Discussion

In this study, the bacterial cell counts in the sputum sample from NMD patients with LRTI serially decreased after starting antimicrobial treatment if the initial therapy was effective. In most patients who saw a therapeutic effect, the most predominant bacterium detected by a clone library analysis in the sputum samples obtained before therapy was almost undetectable by third day of treatment. To our knowledge, there have been no studies evaluating the efficacy of the antimicrobial therapy based on the clinical outcomes and results of bacterial gene-based analyses. An increase in bacterial loads in the sputum was associated with the development of LRTIs (Johansson et al., 2010; Gadsby et al., 2016). On the other hand, our previous study indicated that bacterial cell counts in NMD patients were high even under asymptomatic conditions (median: 10^7 cells /mL) (Ogawa et al., 2019). In the present study, the bacterial cell counts gradually returned to the asymptomatic levels after effective antimicrobial therapy for LRTI. In addition to the disappearance of the most predominant bacterium, the longitudinal analysis of bacterial cell counts is considered useful for evaluating the efficacy of antimicrobial therapy, even if the decrease in the cell count may not be substantial.

Table 3
Serial changes of the proportion of the most predominant bacterium detected by a clone library analysis in the sputum sample obtained before starting antimicrobial treatment.

Case and episode	The most predominant bacterium before starting antimicrobial treatment	Proportion of the bacterium[a] detected by a clone library analysis (%)		
		Before treatment	second day of illness	third day of illness
1–1	*Pseudomonas aeruginosa*	31.0	28.9	80.5
1–2	*Mannheimia granulomatis*	28.6	0	0
2–1	*Pseudomonas aeruginosa*	98.6	0	79.1
3–1	*Pseudomonas aeruginosa*	14.1	8.14	89.7
4–1[b]	*Haemophilus influenzae*	82.6	93.4	82.6
4–2	*Moraxella catarrhalis*	40.9	0	0
5–1	*Streptococcus oralis*	27.7	44.6	0
6–1	*Haemophilus influenzae*	98.9	86.3	65.2
7–1	*Neisseria cinerea*	37.8	0	0
8–1	*Haemophilus influenzae*	73.6	16.5	0
8–2	*Haemophilus influenzae*	57.9	0	0
8–3	*Haemophilus influenzae*	58.2	75.0	0
9–1	*Streptococcus oralis*	22.1	31.8	12.9

[a] The bacterium detected by a clone library analysis before starting antimicrobial treatment.
[b] Clinical symptoms and laboratory data improved not following initial treatment but after the change of antimicrobial therapy.

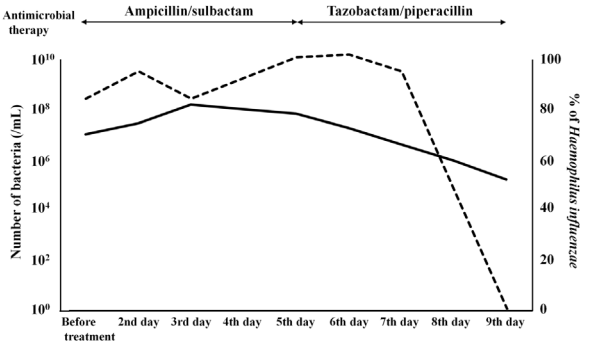

Figure 2. Serial changes of the total bacterial cell count (solid line, left y-axis) and the proportion of the most predominant bacterium detected by a clone library analysis in the sputum sample obtained before antimicrobial treatment (broken line, right y-axis) in the patient who was refractory to initial antimicrobial therapy. The numbers of bacteria stained by ethidium bromide were counted in part of each sputum sample (before DNA extraction was performed).

The Japanese guideline for the treatment of pediatric LRTI recommends around five days of penicillin therapy for previously-healthy children with LRTI (Mikasa et al., 2016). However, as drug-resistant bacteria colonize the respiratory tract and cause severe LRTI in NMD patients because of their difficulty clearing sputum from their airways (Millman et al., 2016; Thorburn et al., 2009), longer-term treatment with broad-spectrum antimicrobial agent tends to be preferred. In a previous study that evaluated the efficacy of antimicrobial therapy by clinical and laboratory outcomes, the proportion of the treatment failure was higher in children with community-acquired pneumonia receiving 3-day antimicrobial therapy than in those receiving 5- or 10-day therapy whereas 5-day therapy was not inferior to 10-day therapy (Greenberg et al., 2014). Given the results of that previous and the present studies, the number of patients with treatment failure may have increased if we had stopped antimicrobial therapy immediately after the disappearance of the most predominant bacterium in the clone library analysis. However, the duration of antimicrobial therapy sufficient for LRTI in NMD patients may be at least the same as that in previous-healthy patients (5-day treatment), although the optimal duration of the therapy may be difficult to determine based on our results alone.

A previous study indicated that around 5 days of penicillin therapy was also clinically effective for bacterial LRTI in most NMD patients (Kawamura et al., 2018). The results of this study indicated for the first time from a microbiological perspective that short-term penicillin therapy, such as that with ampicillin/sulbactam, recommended in the guideline for LRTI in previously-healthy children in Japan was sufficiently effective, even for LRTI in NMD patients.

In a patient who developed acute pneumonia due to β-lactamase-producing ampicillin-clavulanate-resistant *H. influenzae*, the clinical symptoms did not improve, and the total bacterial cell counts did not decrease by the third day of treatment with ampicillin/sulbactam (Figure 2). The efficacy of antimicrobial therapy is generally evaluated 48–72 h after starting the therapy, and changes to the initial therapy are considered after the re-evaluation (Mikasa et al., 2016). Although only one patient was analyzed, from a bacteriological point of view, switching to other antimicrobial agent needs to be considered in patients who fail to show a therapeutic effect 72 h after starting therapy because, in this study, the most predominant bacterium detected by a clone library analysis in the sputum samples obtained before therapy was almost undetectable by third day of treatment. In addition, the bacterial cell counts in sputum, which are important for the diagnosis of bacterial LRTIs (Gadsby et al., 2016), may be useful for evaluating the therapeutic effect.

In one episode that showed a therapeutic effect (No. 1-1), neither the total bacterial cell counts nor the proportion of the most predominant bacterium in the sputum samples obtained before therapy decreased after therapy. Furthermore, in one episode (No. 3-1), the proportion of the most predominant bacterium in the sputum sample obtained before therapy increased after therapy although the total bacterial cell counts had decreased. *P. aeruginosa* was detected from these samples as the most predominant bacterium before therapy. Since the clinical symptoms improved following the administration of antimicrobial agents without activity against the bacterium in many NMD patients with colonization of *P. aeruginosa* in the lower respiratory tract (Kawamura et al., 2018; Ogawa et al., 2019), the most predominant bacterium detected from pre-treatment sputum samples may not have been the causative pathogen in this patient. Alternatively, LRTI in this patient might have improved spontaneously, as respiratory viruses are frequently detected from samples obtained in an infectious state in NMD patients (Johansson et al., 2010; Ogawa et al., 2019).

This study has some limitations. First, it was impossible to completely amplify all of the bacterial 16S rRNA genes with the universal primers that were used in this study. The sensitivity of

the primers for the bacterial species registered in the Ribosomal Database Project II database was approximately 92%. However, the bacteria that were not detectable using these primers do not include pathogenic bacteria. Second, there was no method to evaluate whether the bacteria that were predominantly identified by a 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 (Gadsby et al., 2016; Johansson et al., 2010). Our hypothesis that predominant bacteria would be causative pathogens may be justified. However, it is also necessary to consider that multiple bacteria may cause LRTI especially in episodes with a low proportion of the most predominant bacterium. Third, the clone library analysis method has more technical limitations than the metagenome sequencing analysis. The clone library analysis has an advantage in that it can precisely identify bacteria at the species level because of the relatively long length of sequences compared to next-generation sequencing (Kawanami et al., 2011; Pérez-Losada et al., 2018). We performed a clone library analysis in this study because it was the most clinically efficient method of identifying the causative bacteria at the species level. Finally, study population was relatively small; this could have affected the accuracy of the statistical analysis.

In conclusion, from a microbiological perspective, it was suggested that the optimal duration of antimicrobial therapy for LRTI in NMD patients was the same as that in previously-healthy children if the initial therapy was shown to be effective. The appropriate antimicrobial therapy is needed in order to prevent an increase in multi-drug-resistant bacteria-associated LRTI (Elias et al., 2017; WHO, 2015). Further large-scale studies using this method should be performed in order to determine the optimal antimicrobial therapy for NMD patients with LRTI.

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Ethical approval

Our study was approved by the Institutional Review Board of the University of Occupational and Environmental Health, Japan.

Conflict of interests

None.

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