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Original Article

Evaluation of the usefulness of culture of induced sputum and the optimal timing for the collection of a good-quality sputum sample to identify causative pathogen of community-acquired pneumonia in young children: A prospective observational study

Masato Ogawa [a,b], Takayuki Hoshina [a,*], Asmaa Abushawish [a], Koichi Kusuhara [a]

[a] *Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan*
[b] *Department of Pediatrics, Kitakyushu General Hospital, Japan*

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Geckler classification;
Induced sputum culture

Abstract *Background:* The usefulness of an induced sputum in the identification of causative bacteria of community-acquired pneumonia (CAP) in young children is controversial. This study aimed to investigate the significance of the implementation of an induced sputum culture among children with CAP and the impact of prior use of antimicrobial agents on the quality of the sample and result of the culture.
Methods: This prospective study included 96 children hospitalized for acute bacterial CAP whose sputum samples were collected by suctioning from the hypopharynx through the nose. The samples were evaluated for their quality using Geckler classification, and the result of this conventional culture method was compared to that of a clone library analysis of the bacterial 16S rRNA gene sequence for each sample.
Results: The concordance between bacteria isolated by sputum culture and the most predominant bacteria identified by a clonal library analysis was significantly higher in the samples judged as a good quality (Geckler 5, 90%) than in others (70%). The rate of good-quality sputum sample was significantly higher in samples collected from patients without prior antimicrobial therapy (70%) than in those from patients with it (41%). The concordance between the two methods was significantly higher in the former (88%) than in the latter population (71%).

* 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).

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Conclusion: Bacteria isolated by the culture using good-quality sputum samples collected from children with CAP were more likely to be causative pathogens. Sputum samples collected before starting antimicrobial therapy showed better quality and higher probability of the identification of causative pathogens.
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Introduction

The identification of causative pathogens of acute pneumonia in children has been considered to be more difficult than that in adults, because children, especially those that are young children, cannot expectorate sputum by themselves.[1] In children, causative pathogens of pneumonia can be determined by isolating pathogens from blood, pleural effusion or bronchial lavage fluid.[1–4] However, the frequency of the isolation of bacterium from blood is very low (<10%)[2,3] and pleural effusion and bronchial lavage fluid are collected by invasive procedures that require technical expertise not available in many medical institutions.[4] Recently, there has been an increasing argument for the importance of induced sputum collection to identify causative pathogens of pneumonia among children.[5] We previously indicated that, in children with lower respiratory tract infection, 70% of sputum samples suctioned from the hypopharynx through the nose were suitable for bacterial examination and that the identification of causative bacteria were successful in 62% of the samples, suggesting that it was useful to collect sputum by a relatively non-invasive procedure for the identification of the causative pathogen of pneumonia in children.[6] On the other hand, there was a report that culture of induced sputum samples was not useful as a diagnostic tool for pneumonia in young children.[7] It may be necessary to assess the usefulness of sputum culture with alternative methods, because these previous studies evaluated its usefulness only through conventional culture methods.[6,7] Furthermore, the assessment of the optimal timing to collect a good-quality sputum sample is also needed because prior use of antimicrobial agents may affect the quality of sputum.[5,8]

In this study, to investigate the usefulness of sputum culture in the identification of causative pathogens of community-acquired pneumonia (CAP) in young children, we analyzed sputum samples suctioned from the hypopharynx through the nose of patients with CAP, and compared the results of a conventional culture method with those of a clone library analysis of the bacterial 16S rRNA gene sequence, a prevailing method in bacterial examination.[9] Moreover, we investigated the optimal timing to collect a good-quality sputum sample, in terms of the impact of prior antimicrobial use on the quality of sample.

Materials and methods

Study population

This prospective study enrolled 96 previously healthy children admitted to the Department of Pediatrics, the Hospital of the University of Occupational and Environmental Health, Japan and Kitakyushu General Hospital from May 2017 to August 2021 for acute bacterial CAP whose sputum samples were collected by suctioning from the hypopharynx through the nose on admission. Pneumonia was diagnosed when the chest X-ray showed consolidation and/or infiltration, in addition to the presence of worsening of cough and sputum production, accompanied by abnormal breath sounds on auscultation. The presence or absence of abnormalities on chest X-ray films was assessed by two of the authors (M.O. and T.H.). Either peripheral white blood cell counts $>12 \times 10^9$/L or serum C-reactive protein level >20.0 mg/L or both were used for the suspicion of bacterial infection.[10] The clinical information of the patients was collected using a standardized case report form.

Sample collection, bacteriological examination, and the evaluation of sputum quality

A sputum sample was collected from patients by inserting a collection tube into the patient's nose to the hypopharynx. Part of each sputum sample was processed for Gram staining and bacterial culture. These procedures were performed routinely in the bacteriology laboratory of our hospitals. The remainder of the samples was stored at -20 °C for the bacterial gene analysis. The quality of sputum sample was judged from Geckler classification. Gram stain smears, classified as Geckler class 5, were judged to be suitable for bacterial examination. Bacterial culture was performed on all collected samples regardless of the result of the Gekcler classification. We compared the results of bacterial analyses between the samples judged as Geckler 5 and others.

Definition of presumptive causative pathogen of CAP

Using only sputum samples judged as Geckler class 5, we determined the definition of the presumptive causative pathogen of CAP. In conventional culturing method, when phagocytized bacterial cells were seen on the Gram stain smear of the sputum sample and corresponding bacterium was isolated later, it was identified as the presumptive causative bacterium. Furthermore, in the result of the clone library analysis performed using the sputum sample, the most predominant bacterium identified by the analysis was defined as the presumptive causative bacterium. When phagocytosis of multiple bacteria (eg. gram-positive cocci and gram-negative rods) were seen on the Gram stain smear of the sputum sample and corresponding bacteria was isolated later, and the proportion of two bacteria

identified by the clone library analysis were subsequently high and close (the difference was <20%), we judged as polymicrobial infection.

Bacterial cell counts and cell lysis efficiency analysis

Each sputum sample (100 μL) and 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] were mixed and the mixture was left for 10 min at room temperature. After filtering the mixture through a 0.2-μm-pore filter (Millipore, Bedford, MA), bacteria on the filter were counted using a microscope in randomly chosen 30 fields and the mean number of bacteria per milliliter was calculated. To confirm the efficiency of cell lysis, the number of remaining bacteria after DNA extraction was calculated by the same method. Cell lysis efficiency was calculated using the following expression [100 − (post-extraction number/pre-extraction number) × 100]. To eliminate bias due to the bacteriolysis rate, we judged samples with a cell lysis efficiency >80% as suitable for further analysis (Fig. 1).

DNA extraction

The extraction of DNA from sputum samples was performed by vigorous shaking together with sodium dodecyl sulfate solution (final concentration, 3.0%) and glass beads as previously reported.[11]

PCR amplification of the 16S rRNA gene

A Veriti thermocycler (Applied Biosystems, Foster City, CA, USA) was used for the amplification of the 16S rRNA gene. The mixtures with the universal primer set (E341F: 5′-CCTACGGGAGGCAGCAG-3′ and E907R: 5′-CCGTCAATTCMTT-TRAGTTT-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 subsequently one cycle for the final elongation step at 72 °C for 7 min (Fig. 1).

Clone library analysis and determination of nucleotide sequences

The amplified PCR products were cloned into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), and then the nucleotide sequences of 96 randomly selected colonies from each clone library were determined by a sequencing analysis (Fig. 1). The partial fragments of the cloning vectors (pCR 4) with the inserted PCR products were amplified using the AmpliTaq 360 Master Mix, GC Enhancer, and Pre-Seq primer set (F; 5′-GTTTTCCCAGT-CACGACG-3′ and R; 5′-CAGGGAACAGCTATGAC-3′, Applied Biosystems). After eliminating the primers and deoxyribonucleotided triphosphate from the PCR mixture using an Exonuclease I and Alkaline Phosphatase (Shrimp) (TaKaRa Bio Inc., Otsu, Shiga, Japan), a 1 μL aliquot was used as the template for sequencing reaction. The sequencing reactions were accomplished by mixing with primers M13F and the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The nucleic acid sequences were analyzed by a 3130 × l Genetic Analyzer (Applied Biosystems).

Homology searching

The Geneious prime software program (Biomatters Ltd., Auckland, New Zealand) was used to check the quality and

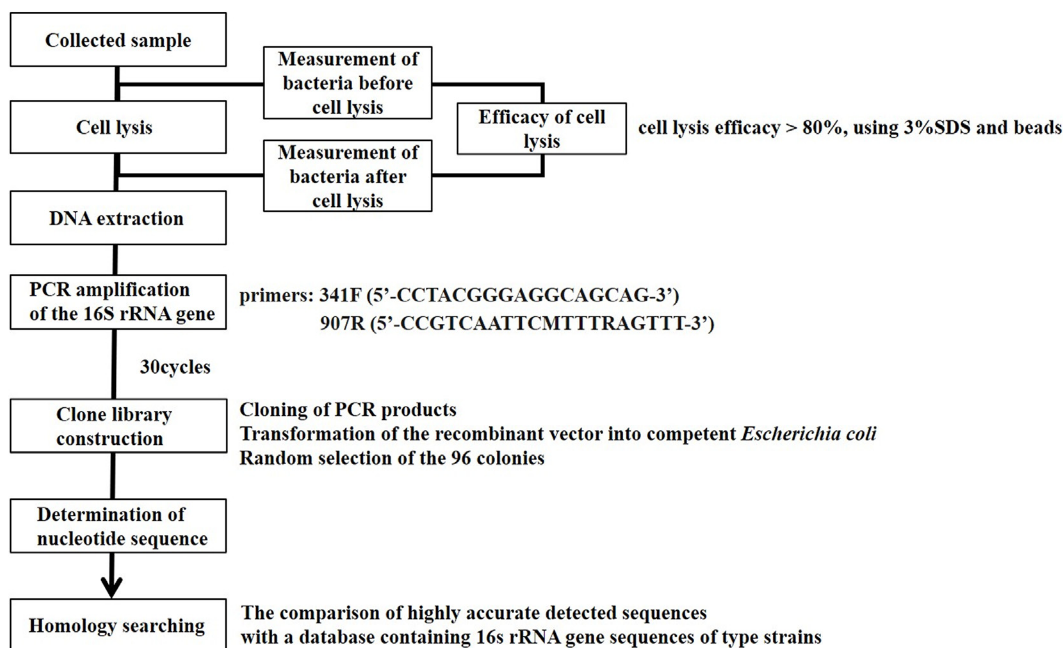

Figure 1. Flow chart of the analysis of the bacterial 16S rRNA gene.

trimming of the sequences. Among the sequences remaining after the quality check, only the reads containing precise sequences of the universal primers E341F and E907R were selected. In addition, the raw data were checked, and sequences including ambiguous bases between the primer sequences were excluded. The remaining highly accurate sequences were trimmed to remove primer and vector sequences. The comparison of highly accurate detected sequences with a database containing 16s rRNA gene sequences of type strains was performed using a basic local alignment search tool algorithm (blast) (Fig. 1). A phylotype sharing >97% homology compared with the sequence of the type strain was considered a presumptive species in the present study.

Statistical analyses

The statistical analyses were performed to use the SPSS statistics software program (version 21; SPSS Inc., Chicago, IL, USA, and IBM, Armonk, NY, USA). The differences between the quantitative values in the bacteriological analysis were compared by the Wilcoxon signed-rank test. *P*-values <0.05 were considered statistically significant.

Ethical approval

Our study was approved by the Institutional Review Board of the University of Occupational and Environmental Health (H28-242) and Kitakyushu General Hospital (H29-2), Japan.

Results

Characteristics of patients with bacterial CAP

The demographic and clinical characteristics of the eligible children are shown in Table 1. The median age of the 96 patients was 12 months old (range: 3—168). Almost all patients had fever (99%) and cough (95%). Twenty-nine patients (30%) received oral antimicrobial therapy before admission. Approximately half of them (48%) took oral cephalosporins. After admission, many patients (69%) were initially treated with ampicillin or ampicillin/sulbactam. Almost all, excluding one patient, improved following the initial antimicrobial therapy. The median duration of antimicrobial therapy was 5 days. Approximately half of the patients (49%) required supplemental oxygen, whereas none of the patients required mechanical ventilation. None died from bacterial CAP.

The screening of some viruses was performed when the attending physician deemed it necessary. A total of 77 patients underwent at least one virological test (rapid antigen detection test for respiratory syncytial virus, human metapneumovirus, influenza virus or adenovirus, or multiplex PCR assay for the detection of respiratory viruses), and 28, 3, 2 and 2 of them were positive for respiratory syncytial virus, adenovirus, human metapneumovirus and parainfluenza virus type 3, respectively. Nine of the 35 patients (26%) received oral antimicrobial therapy before admission. Even in all these patients, their symptoms rapidly improved after the appropriate antimicrobial therapy.

Table 1 The demographic and clinical characteristics of the eligible children.

Characteristics	Eligible patients, n = 96
Age, months, median (range)	12 (3—168)
Gender, n, %male	38 (40)
Fever*[1], n (%)	95 (99)
Cough, n (%)	91 (95)
Antimicrobial therapy before admission, n (%)	29 (30)
Oral antimicrobial agent taken before admission	
Amoxicillin or amoxicillin/clavulanate, n (%)	6 (21)
Cephalosporin*[2], n (%)	14 (48)
Macrolide, n (%)	5 (17)
Quinolone*[2], n (%)	3 (10)
Unknown, n (%)	1 (3)
White blood cell count, 10^9/L, median (range)*[3]	12.5 (6.5—30.9)
Serum C-reactive protein level, mg/L, median (range)*[3]	44.5 (0.4—254.0)
Antimicrobial agent taken after admission*[4]	
Ampicillin or ampicillin/sulbactam*[5], n (%)	67 (69)
Cephalosporin*[5], n (%)	28 (29)
Macrolide, n (%)	2 (2)
Supplemental oxygen, n (%)	47 (49)
Mechanical ventilation, n (%)	0 (0)
Patients changed to other antimicrobial agents from the initial one, n (%)	1 (1)
Duration of antimicrobial therapy, days, median (range)	5 (2—10)
Duration of hospitalization, days, median (range)	6 (2—19)
Fatal case, n (%)	0 (0)

*[1]It included a febrile patient >38.5 °C.
*[2]One patient was initially treated with cephalosporin and changed to quinolone.
*[3]Either peripheral white blood cell counts $>12 \times 10^9$/L or serum C-reactive protein level >20.0 mg/L or both were used for the suspicion of bacterial infection. All patients fulfilled one or both of these two criteria.
*[4]All patients were treated with intravenous antimicrobial agents.
*[5]One patient was initially treated with ampicillin and changed to cefotaxime.

Accuracy of the sputum culture in presuming causative bacterium of pneumonia based on sample quality

Of the 96 collected samples, 59 (61%) were judged as Geckler 5, whereas the remaining 37 (39%) were judged as Geckler 1—4 or 6. The concordance between the bacteria identified by the sputum culture and most predominant bacterium identified by the clonal library analysis was significantly higher in the samples judged as Geckler 5 (90%) than in others (70%, $P = 0.029$) (Table 2). In the samples judged as Geckler 5 for which most predominantly isolated

bacteria by culture were consistent with the most predominant one identified by a clone library analysis, the presumed causative bacterium were *Haemophilus influenzae* (53%, n = 28), *Streptococcus pneumoniae* (30%, n = 16) and *Moraxella catarrhalis* (25%, n = 13). The proportion of the most predominant bacterium among the 96 clones in the clone library analysis was also significantly higher in the samples judged as Geckler 5 than in others (P = 0.030) (Table 2, Fig. 2). These results suggested that the causative bacteria of CAP in children may be likely to be identified by culture if a good-quality sputum sample can be collected.

In 77 patients undergoing the virological test, we also compared the results of sputum sample analyses between children with and without viral infections. The rate of a good-quality sputum sample was significantly higher in samples collected from patients with viral infections (27/35, 88%) than in those from patients without them (21/42, 50%) (P = 0.014). As for the concordance between bacterium predominantly isolated by sputum culture and the most predominant bacterium identified by the clonal library analysis, there was no significant difference between the 2 groups (89% vs. 79%).

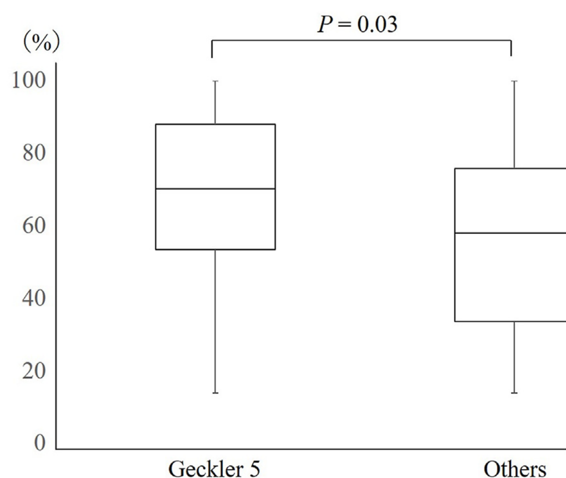

Figure 2. The comparison of the rate of the most predominant bacterium among 96 colonies in the clone library analysis between samples judged as Geckler 5 and Geckler 1—4 or 6. The bottom and the top of the line correspond to 25th and 75th percentile points, respectively. The line within the box represents median.

Impact of prior antimicrobial therapy on the quality of the sputum sample and the accuracy of the culture in presuming causative bacterium of pneumonia

Of the samples collected from 29 patients with CAP receiving oral antimicrobial agents before admission, only 12 (41%) were judged as Geckler 5. In contrast, 47 of the 67 samples (70%) collected from patients without prior antimicrobial therapy were judged as Geckler 5 (Table 3). The rate of a good-quality sputum sample was significantly higher in samples collected from patients without prior antimicrobial therapy than in those from patients with it (P = 0.007). The concordance between bacterium predominantly isolated by sputum culture and the most predominant bacterium identified by the clonal library analysis was significantly higher in the former (88%) than in the latter population (71%, P = 0.042) (Table 3). The same tendency was seen when limited to the samples judged as Geckler 5, although not to a significant extent (Table 4). The proportion of the most predominant bacterium among 96 clones in the clone library analysis was also significantly higher in the samples collected from patients without prior antimicrobial therapy than in those from patients with it (P = 0.024) (Table 3). The same tendency was seen when the samples were limited to Geckler 5 samples, although not to a significant extent (Table 4). These results suggested that, in children with CAP, the sample may be likely to be a good quality and the causative bacteria may be likely to be identified by the culture if the sample before starting antimicrobial therapy can be collected.

Table 2 The comparison of the analysis results of sputum samples in children with community-acquired pneumonia based on the quality of the collected samples.

	Geckler 5 n = 59	Others*[1] n = 37	P-value
Concordance between the results of the culture and the clone library analysis*[2], n (%)	53 (90)	26 (70)	0.029
Proportion of the most predominant bacterium in the clone library analysis*[3], %, median (range)	68 (14—100)	54 (6—100)	0.030

*[1]Geckler 1—4 and 6 are included in this category.
*[2]This indicates that bacteria isolated by sputum culture were consistent with the most predominant bacterium identified by the clone library analysis.
*[3]This indicates the proportion of the clones that were the most predominant bacterium among 96 clones.

Characteristics of the samples with discordance of the results between the clone library analysis and the conventional culturing method

The results of the clone library analysis and the conventional culturing method were discordant in 18 of the 96 samples. In all 11 of the 18 samples judged as Geckler 1—4 or 6, URT commensal bacteria that are difficult to be identified by the conventional culturing method were predominantly identified by the clone library analysis (Table 5). In three of the seven samples judged as Geckler 5, these bacteria were predominantly identified (Table 5). Of

Table 3 The comparison of the quality of the collected samples and the analysis results of the samples between patients with and without prior antimicrobial therapy.

	Prior antimicrobial therapy (+) n = 29	Prior antimicrobial therapy (−) n = 67	*P*-value
Samples judged as Geckler 5, n (%)	12 (41)	47 (70)	0.007
Concordance between the results of the culture and the clone library analysis*[1], n (%)	20 (71)	58 (88)	0.042
Proportion of the most predominant bacterium identified by the clone library analysis*[2], %, median (range)	51 (8—100)	67 (9—100)	0.024

*[1]This indicates that bacteria isolated by sputum culture were consistent with the most predominant bacterium identified by the clone library analysis.
*[2]This indicates the proportion of the clones that were the most predominant bacterium among 96 clones.

Table 4 The comparison of the quality of the collected samples and the analysis results of the samples between patients with and without prior antimicrobial therapy in the samples judged as Geckler 5.

	Prior antimicrobial therapy (+) n = 12	Prior antimicrobial therapy (−) n = 47	*P*-value
Concordance between the results of the culture and the clone library analysis*[1], n (%)	9 (75)	43 (91)	0.11
Proportion of the most predominant bacterium identified by the clone library analysis*[2], %, median (range)	61.4 (14—99)	71.9 (14—100)	0.50

*[1]This indicates that bacteria isolated by sputum culture were consistent with the most predominant bacterium identified by the clone library analysis.
*[2]This indicates the proportion of the clones that were the most predominant bacterium among 96 clones.

the remaining four samples, one was collected from the patient with antimicrobial therapy before admission. In three samples collected from the patients without preceding antimicrobial therapy, *H. influenzae* (n = 2) and *M. catarrhalis* (n = 1) were predominantly identified by the clone library analysis while these results did not correspond to those of the conventional culturing method (Table 5). The reason of this discordance was uncertain.

Discussion

In this study, we investigated whether sputum samples suctioned from the hypopharynx through the nose were suitable for the identification of causative bacteria of CAP in children by comparing the results of a conventional culture method and a genetic analysis of the bacterial 16S rRNA gene. A previous study indicated that the collection of sputum sample was difficult in children because they cannot cough up by themselves and that the contamination of upper respiratory tract secretions might mislead the result of the culture.[5] However, many children enrolled in the study had already been taking antimicrobial agents before collecting a sputum sample. In contrast, our study indicated that a good-quality sputum sample (Geckler 5) might be useful to identify causative bacteria of CAP in children and sample collection before starting antimicrobial therapy would be desired for better quality sputum and higher probability of the identification of causative pathogens. Pediatricians should keep in mind to collect good-quality sputum sample from children with CAP prior to antimicrobial therapy.

The quality of a sputum sample is an important factor on the interpretation of the result of the culture and Geckler classification is usually used for the evaluation. Gram stain smear classified as Geckler 4 or 5 is judged to be suitable for bacterial examination.[12] In this study, only proportion of sputum samples judged as Geckler 5, recognized as the highest quality, was investigated to strictly evaluate the accuracy of sputum culture in the identification of causative bacteria of CAP in children. The nebulization with sterile hypertonic saline has been considered to be a useful procedure for collecting a good-quality sputum,[5] whereas good-quality samples could be collected from >60% of children with CAP even by a simple method of suctioning sputum from the hypopharynx through the nose.[6] In addition to the method of collecting sample, prior use of antimicrobial agent also has significant effects on the result of sputum culture and the quality of sputum. Antibiotic exposure was associated with the reduction of yield from induced sputum culture.[8,13,14] The use of an antimicrobial agent can reduce the amounts of pathogenic bacteria in sputum samples,[15] and the amounts of bacteria colonizing in the oral cavity or upper respiratory tract may relatively increase. Furthermore, the reduction of the amounts of pathogenic bacteria in sputum samples may lead even the reduction of the amount of sputum,[16] resulting in a relative increase in the proportion of upper respiratory secretions. The results of our study were also considered to reflect the impact of prior use of antimicrobial agent on the quality of sputum and the identification of the causative bacteria of CAP in children.

A recent regional epidemiological study in Japan showed that *H. influenzae* was the most prevalent bacterial

Table 5 Bacteria identified in the samples with discordance of the results between the clone library analysis and the conventional culturing method.

Sample No.	Geckler's classification	Preceding antimicrobial therapy	Clone library analysis		Conventional culturing method
			Predominant bacteria*[1]	% of clones	Identified bacteria
1	5	Yes	*R. amarae*	92	*S. pneumoniae*
					M. catarrhalis
2	5	Yes	*H. influenzae*	68	*K. pneumoniae*
3	5	No	H. influenzae	61	URT commensal bacteria*[2]
			S. pseudopneumoniae	14	
4	5	No	*S. pseudopneumoniae*	29	URT commensal bacteria*[2]
			P. veroralis	12	
5	5	No	*M. catarrhalis*	54	*K. pneumoniae*
			S. pseudopneumoniae	29	*S. marcescens*
6	5	No	*H. influenzae*	50	*S. pneumoniae*
			N. lactamica	17	*S. aureus*
7	5	No	*N. perflava*	94	*H. influenzae*
8	Others*[3]	Yes	*P. monteilii*	32	None
			C. propinquum	31	
			P. plecoglossicida	20	
9	Others*[3]	Yes	*S. peroris*	48	None
			S. oralis	13	
10	Others*[3]	Yes	*R. amarae*	30	None
11	Others*[3]	Yes	*V. dispar*	13	URT commensal bacteria*[2]
			P. melaninogenica	11	
			S. pseudopneumoniae	10	
12	Others*[3]	Yes	*N. perflava*	29	*S. aureus*
			N. cinerea	23	
			S. oralis	16	
13	Others*[3]	Yes	*S. cristatus*	52	*S. pneumoniae*
			G. taiwanensis	29	
			F. nucleatum	15	
14	Others*[3]	No	*S. pseudopneumoniae*	14	None
			S. oralis	14	
			C. argentoratense	13	
15	Others*[3]	No	*C. propionquum*	33	URT commensal bacteria*[2]
			M. catarrhalis	19	
16	Others*[3]	No	*H. aegyptius*	76	URT commensal bacteria*[2]
			S. oralis	16	
17	Others*[3]	No	*H. aegyptius*	33	URT commensal bacteria*[2]
			S. cristatus	28	
			M. lincolnii	15	
18	Others*[3]	No	*R. amarae*	25	*S. pneumoniae*
			S. warneri	24	*H. influenzae*

*[1]Only top three bacteria or bacteria with >10% of the detection rate was shown.
*[2]The species could not be identified.
*[3]The samples judged as Geckler 1–4 or 6 were included. URT: upper respiratory tract, *C. argentoratense*: *Corynebacterium argentoratense*, *C. propinquum*: *Corynebacterium propinquum*, *F. nucleatum*: *Fusobacterium nucleatum*, *G. taiwanensis*: *Gemella taiwanensis*, *H. aegyptius*: *Haemophilus aegyptius*, *H. influenzae*: *Haemophilus influenzae*, *K. pneumoniae*: *Klebsiella pneumoniae*, *M. catarrhalis*: *Moraxella catarrhalis*, *M. lincolnii*: *Moraxella lincolnii*, *N. cinerea*: *Neisseria cinerea*, *N. lactamica*: *Neisseria lactamica*, *N. perflava*: *Neisseria perflava*, *P. melaninogenica*: *Prevotella melaninogenica*, *P. veroralis*: *Prevotella veroralis*, *P. monteilii*: *Pseudomonas monteilii*, *P. plecoglossicida*: *Pseudomonas plecoglossicida*, *R. amarae*: *Rothia amarae*, *S. marcescens*: *Serratia marcescens*, *S. aureus*: *Staphylococcus aureus*, *S. cristatus*: *Streptococcus cristatus*, *S. oralis*: *Streptococcus oralis*, *S. peroris*: *Streptococcus peroris*, *S. pneumoniae*: *Streptococcus pneumoniae*, *S. pseudopneumoniae*: *Streptococcus pseudopneumoniae*, *S. warneri*: *Streptococcus warneri*, *V. dispar*: *Veillonella dispar*.

pathogen isolated from the sputum of hospitalized children for bacterial CAP.[17] Although the incidence of pneumococcal pneumonia in children decreased after the introduction of pneumococcal conjugate vaccine in the national immunization program in Japan, *S. pneumoniae* was still the second leading cause of bacterial CAP.[17] Furthermore, *M. catarrhalis* was also a major causative pathogen of CAP.[17] The same tendency was also observed in our study.

Although the high incidence of β-lactamase-nonproducing ampicillin-resistant *H. influenzae* in Japan is concerned,[18] based on these result, intravenous ampicillin therapy is recommended for the initial treatment for pediatric CAP with reference to the Japanese guideline for the treatment of pediatric CAP.[19]

It is difficult to determine causative bacteria of pneumonia when multiple bacteria are isolated by sputum culture. Furthermore, in the genetic analysis of the bacterial 16S rRNA gene, there is no universal definition of pneumonia due to multiple bacteria. We determined our own definition of pneumonia due to multiple bacteria. Based on the criteria, four of the 53 patients (7.5%) were considered as polymicrobial infection. In the previous studies investigated using induced sputum samples collected by methods other than the one we tried,[20,21] the incidence of pneumonia due to multiple bacteria was around 10%. As these results were similar to that of our study, we believed that induced sputum collected by the method we used was also useful to identify polymicrobial infection.

In this study, to assess the accuracy of the results of induced sputum culture, we also performed a clone library analysis of the bacterial 16S ribosomal RNA gene sequence using the same sample and investigated whether bacteria predominantly isolated by the culture were consistent with the most predominant ones identified by the clonal library analysis. Consequently, the higher concordance rate between the results of the two methods was shown in a good-quality sputum sample. Molecular methods, such as nucleic acid amplification tests, have the potential to improve the accuracy of the identification of causative bacteria in pneumonia.[15,20,22] Many previous studies mainly identified causative pathogens in CAP with the PCR amplification of the pathogen-specific genes.[7,15,20] The clone library analysis has the advantages in that it can evaluate the proportion of each bacterial phylotype in each sample in addition to the ability to detect all bacterial phylotypes, and has been useful for the identification of causative pathogens in respiratory disorders.[9,22] Although no definitive interpretations regarding the results of a clone library analysis have been established, we believe that the method is useful for the verification of the diagnostic accuracy of a conventional culture.

This study was associated with some limitations. First, it is impossible to amplify the all bacterial 16S rRNA genes completely. The sensitivity of the primers used in this study for the bacterial species was approximately 92%, but not all pathogenic bacteria could be detected by using these primers.[22] Second, there was no method to evaluate whether the bacteria that were identified by the 16S rRNA genes clone library analysis predominantly were real causative pathogens of CAP. But, in general, an elevation in the bacterial load in sputum is associated with the development of bacterial pneumonia. So, our hypothesis that predominant bacteria would become real causative pathogens may be justified. Furthermore, patients in whom virus was detected may not have had bacterial CAP. However, as their symptoms rapidly improved after the appropriate antimicrobial therapy, we believed that all patients clinically had bacterial CAP. Third, the clone library analysis has more technical limitations than the next-generation sequencing analysis. The clone library analysis is superior to next-generation sequencing in that it can exactly identify bacteria at the species level because of the relatively long lengthy of sequences.[7,23] As it was the most clinically efficient method of identifying the causative bacteria at the species level, a clone library analysis was performed in this study. Finally, the study population was relatively small, which may have affected the accuracy of the statistical analysis.

In conclusion, bacteria isolated by the culture using good-quality sputum samples collected from children with CAP were more likely to be causative pathogens. Sputum samples collected before starting antimicrobial therapy showed better quality and had a higher probability of identifying the causative pathogens than those collected after therapy. A good-quality induced sputum sample may be useful to identify the causative bacteria of CAP in children, and it is desirable to collect samples before initiating antimicrobial therapy.

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