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Case report

Severe respiratory failure due to co-infection with human metapneumovirus and *Streptococcus pneumoniae*

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Keywords:
Human metapneumovirus
Streptococcus pneumoniae
Next-generation sequencer
Real-time PCR
Co-infection

A B S T R A C T

A 64-year-old male patient was admitted with respiratory failure, although chest X-rays revealed only mild bronchiolitis. *Streptococcus pneumoniae*, which usually presents as massive lobular pneumonia, was isolated from sputum, however, pan-pathogen screening using a next-generation sequencer also detected human metapneumovirus genome fragments.

Introduction

It has been reported that Human metapneumovirus (hMPV) was associated with various upper and lower respiratory tract syndromes, including common colds, bronchitis, pneumonia, and asthma exacerbation, with more severe diseases reported for young children, elderly subjects, and immunocompromised patients [1–3].

In adults, large outbreaks of hMPV infection in long-term care facilities (LTCF) have been reported, the magnitude and severity of which were similar to those of outbreaks typically associated with influenza or RS virus infection [4,5]. Boivin et al. reported that 9 (9.4%) of 96 patients in the LTCF died due to respiratory infection, including 3 patients who had confirmed hMPV infection [4].

In this report, we found a severe respiratory failure case due to co-infection hMPV and *Streptococcus pneumoniae* in adult patient, and this case was diagnosed by not only routine microbiological methods and but also genetic analysis, including next-generation sequencer.

Case report

A 64-year-old male patient who had been followed up for mild dilated cardiomyopathy became dyspneic and was admitted to our hospital in March 2013. He had been febrile (37.5 °C) for 1 week and had been coughing and short of breath for 2 days although he was a nonsmoker.

His physiological parameters upon admission were as follows: blood pressure 94/52 mmHg, respiratory rate 24 breaths/min and PaO_2 62 mmHg, despite the immediate administration of supplemental oxygen (10 L/min).

Laboratory studies revealed no leukocytosis (white blood cells, 3500/mL), but its differentiation was as follows: Neu 87.5%, Lym 7.6%, Mono 3.8%, Eo: 0.6%, and Baso 0.4%, respectively, and inflammation was indicated by C-reactive protein 26.79 mg/dL (<0.8 mg/dL). In addition, aspartate transaminase 291 U/L and alanine transaminase 381 U/L; mild renal dysfunction was indicated by creatinine 1.18 mg/dL and blood urea nitrogen59 mg/dL. A normal brain natriuretic peptide (BNP) level of 315.4 mg/mL and barely detectable troponin I (0.09 ng/mL) suggested that heart failure had not worsened and that the patient did not have myocardial infarction.

We isolated *S. pneumoniae*, which usually causes lobular pneumonia and/or bronchopneumonia, from the patient's sputum samples. Gram staining of the sputum also showed typical lancet-shaped Gram-positive diplococcic. We did not isolated *S. pneumoniae* from the blood samples, but the rapid antigen test of

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urine for *S. pneumoniae* (Binax Now, Binax, Portland, USA) was also positive. The serotype of the isolated *S. pneumoniae* was 23F deteremined by Quellung reaction, and the MICs in parentheses indicated that the organism was susceptible to ampicillin (2 μg/mL), ceftriaxon (1 μg/mL), levofloxacin (0.5 μg/mL) and meropenem (0.5 μg/mL) determined by using an automated identification system (MicroScan WalkAway; Siemens, Munich, Germany).

However, in addition to no leukocytosis, chest radiography and computed tomography did not reveal a massive infiltration shadow, but rather showed only very mild bronchiolitis, although the patient required ventilator control after admission (Fig. 1). Therefore, we again considered panbronchiolitis due to atypical pathogens, including mycoplasma, viruses and *Chlamydia*. However, antibodies against *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* were negative, and paired sera did not change. Immunochromatography with Esplain Influenza A&B (Fujirebio Diagnostics Inc., Tokyo, Japan) and Quicknavi-RSV (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) confirmed that he was negative for the influenza virus and RSV antigen, respectively.

Shotgun sequencing of nucleic acids extracted from sputum samples using the MiSeq next-generation DNA sequencer (Illumina, San Diego, CA, USA) as described [6], which was performed routinely for difficult to diagnosis cases in our department, detected a 151-base sequence with 99% similarity to a moiety of the human metapneumovirus (hMPV) genome (Fig. 2). Furthermore, hMPV genes were detected in sputum by regular nested-PCR, and the sequence of this amplicon was reconfirmed as that of hMPV after the extraction of the PCR product. We also confirmed the findings by real-time PCR (Taqman, Light cycler 480, Roche, Basel, Switzerland), which detected 1.9×10^4 copies/mL of the hMPV gene. Diluted antibody for hMPV in his serum was significantly increased to ×10,240, but the rapid antigen detection test (Check hMPV: Meiji Seika Pharma, Tokyo, Japan) was negative.

We diagnosed severe respiratory failure due to panbronchiolitis caused by hMPV and *S. pneumoniae* co-infection. Administration of minocycline (2 × 100 mg/day) and meropenem (3 × 1 g/day) were started because we suspected not only *S. pneumonaie* infection, but also other pathogens including mycoplasma initially. Minocycline was suspended at Day 3, but meropenem was continued for 10 days. These antibacterial therapy and careful respiratory management led to complete recovery.

Discussion

Interactions between viral and bacterial disease are usually interpreted as viral infections predisposing individuals to severe bacterial infections [7,8]. Various mechanisms have been proposed, including virus-induced damage to respiratory cells causing a predisposition to opportunistic bacterial infection or the up-regulation of bacterial adhesion molecules by viral infection [9].

The present study found that exposure to hMPV might modulate *S. pneumoniae* infection. Verkaik et al. screened 57 children aged up to 2 years for colonization with four common respiratory bacterial species associated with seroconversion to hMPV [10]. Whereas a relationship was between exposure to *Haemophilus influenzae*, *Moraxella catarrhalis* or *Staphylococcus aureus* and hMPV seroconversion was not identified, *S. pneumoniae* exposure was significantly associated with increased seroconversion to hMPV. This increase might have been due to increased susceptibility to hMPV infection, increased viral replication or virus spread or enhanced immune responses [10]. The authors concluded from the serological data that either hMPV infection leads to more frequent *S. pneumoniae* carriage or exposure to *S. pneumoniae* increases susceptibility to hMPV infection.

Kukavica-Ibrulj et al. also found that hMPV infection predisposes mice to severe pneumococcal pneumonia [2]. They used an established experimental murine model to validate the hypothesis that hMPV, like influenza virus, increases pneumococcus replication in the lungs and enhances host immunological responses [8]. Secondary bacterial infections often complicate respiratory viral infections, although the mechanisms through which viruses predispose hosts to exacerbated bacterial disease are not completely understood. The most frequently postulated mechanisms include viral destruction of the respiratory epithelium that might increase bacterial adhesion, virus-induced immunosuppression that can lead to bacterial superinfection and an inflammatory response to viral infection that might up-regulate the expression of molecules that bacteria utilize as receptors [2,11].

We found only mild bronchiolitis on chest X-ray and CT images in our patient, although he had symptoms of severe respiratory failure. *S. pneumoniae* usually induces lobular pneumonia or bronchopneumonia when it became a major pathogen. Therefore, hMPV in our patient dominantly infected and induced severe damage to bronchiolar regions, and this process was accelerated by

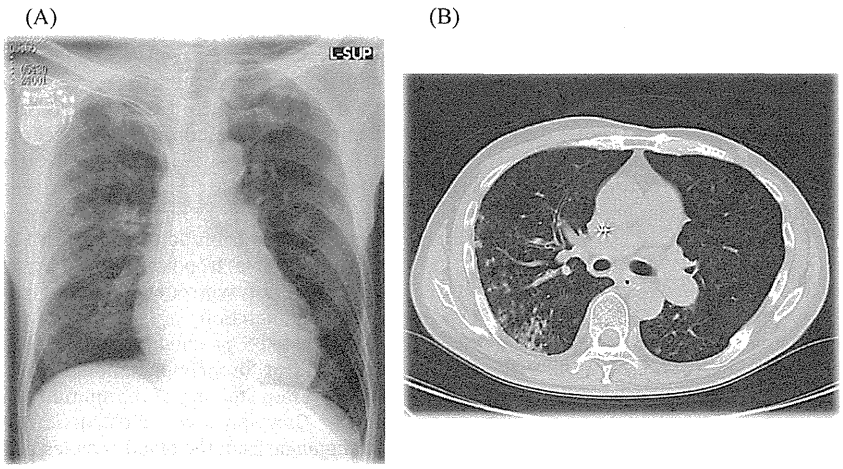

Fig. 1. Chest radiography (A) and computed tomography (B) images upon admission. Absence of consolidation and small multiple lobular shadows in upper and middle fields of right lung suggests bronchiolitis.

Fig. 2. Genetic analysis of hMPV in sputum. Alignment of complete HMPV genome sequence by nucleotide sequence using BLASTN. Reading obtained with MiSeq sequencer (query) was compared with that of HMPV strain Sabana (Subject: NCBI accession number, HM197719.1).

S. pneumoniae infection. Colonizing *S. pneumoniae* might increase hMPV infection and replication, and the number of *S. pneumoniae* also synergistically increased. A significant increase in hMPV antibody during the acute phase also suggested previous hMPV infection followed by *S. pneumoniae* infection in our patient. The mechanisms of severe respiratory failure due to hMPV infection followed by *S. pneumoniae* should be further studied.

We detected hMPV sequences from the patient's sputum and confirmed the findings by real-time PCR, which detected 1.9×10^4 copies/mL of the hMPV gene. These results suggested that such genetic analysis is valuable when patients present with infection caused by hMPV. We did not detect hMPV antigen by immunochromatography using Check hMPV, which usually detects 1×10^6 copies/mL of the hMPV gene [12,13]. The discrepancy between the positive genetic findings and the negative antigen assays also indicates the value of genetic analysis. Although high-throughput next-generation sequencing technologies might be difficult to apply to routine clinical diagnosis, they have potential for clinical genomic studies, as they can exceed the data output of the most sophisticated capillary sequencers based on the Sanger method [14]. We suggest that genetic analysis of samples along with cultures should become routine practice [15].

In conclusion, we described severe respiratory failure caused by co-infection with hMPV and *S. pneumoniae* in a patient whose chest X-ray and CT images showed only mild bronchitis. Genetic analysis, such as next-generation sequencing, revealed hMPV infection. Synergistic effects between hMPV and bacteria should be fully investigated.

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Removal of waterborne pathogens from liver transplant unit water taps in prevention of healthcare-associated infections: a proposal for a cost-effective, proactive infection control strategy

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Abstract

Hospital water supplies often contain waterborne pathogens, which can become a reservoir for healthcare-associated infections (HAIs). We surveyed the extent of waterborne pathogen contamination in the water supply of a Liver Transplant Unit. The efficacy of point-of-use (POU) water filters was evaluated by comparative analysis in routine clinical use. Our baseline environmental surveillance showed that *Legionella* spp. (28%, 38/136), *Pseudomonas aeruginosa* (8%, 11/136), *Mycobacterium* spp. (87%, 118/136) and filamentous fungi (50%, 68/136) were isolated from the tap water of the Liver Transplant Unit. 28.9% of *Legionella* spp.-positive water samples (n = 38) showed high-level *Legionella* contamination ($\geq 10^3$ CFU/L). After installation of the POU water filter, none of these pathogens were found in the POU filtered water samples. Furthermore, colonizations/infections with Gram-negative bacteria determined from patient specimens were reduced by 47% during this period, even if only 27% (3/11) of the distal sites were installed with POU water filters. In conclusion, the presence of waterborne pathogens was common in the water supply of our Liver Transplant Unit. POU water filters effectively eradicated these pathogens from the water supply. Concomitantly, healthcare-associated colonization/infections declined after the POU filters were installed, indicating their potential benefit in reducing waterborne HAIs.

Keywords: Healthcare-associated infection, hospital water supply, infection control, POU water filter, waterborne pathogens
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Introduction

Hospital water supplies have served as reservoirs for waterborne pathogens such as *Legionella* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Mycobacterium* spp. and fungi [1–5]. The degree of the colonization in water supplies has been correlated with the incidence of healthcare-associated infections (HAIs) [6,7]. Forty-two per cent of ICU patients with *Pseudomonas aeruginosa* harbored isolates with identical genotypes to those found in the taps [8]. Water supplies were recognized as one of the most important and controllable, and yet the most overlooked, sources of HAIs [1,2].

Despite water treatment with chlorination, domestic water supplies may still be contaminated by low concentrations of various microorganisms [9]. Although most of the microorganisms are not harmful to the general public, some opportunistic pathogens pose threats to hospitalized patients. In China, the waterborne pathogen contaminations of water supplies have often been overlooked. In fact, the European Working Group for Legionella Infections (EWGLI) reported in 2009 that China was one of the top 15 countries implicated in cases of travel-associated Legion-

naires' disease [10]. In a study of eight hospital water supplies in Shanghai [11], 43.0% (83/193) of water samples were positive for *Legionella* spp., and 63 water samples exceeded the concentration of 10^3 CFU/L. So, we sought to determine if waterborne pathogens were present in the water supply of our hospital, especially in the Liver Transplantation Unit (LTU), where the patients are most susceptible to opportunistic infections. Furthermore, could removal of these waterborne pathogens reduce the incidence rate of hospital-acquired infections in the LTU? Thus, we performed an infection control intervention by: (i) investigating the baseline frequency of waterborne pathogens in the water supply of the LTU, and (ii) evaluating the efficacy of point-of-use (POU) water filters in removing waterborne pathogens. To our knowledge, this is the first environmental surveillance of waterborne pathogens in a hospital water supply in China.

Materials and Methods

Study site

This study was performed in an 18-bed LTU of a university-affiliated general hospital with 1600 beds in Shanghai, China. The Unit consists of nine patient rooms (two patient beds and one sink/tap in each room), one nurses' station and one doctor's office. The hospital receives its water from a municipal water treatment plant without additional on-site disinfection.

Study design

Cold tap water samples were collected between 2009 and 2011 (June, September and October in 2009, January, July, August, September, October and November in 2010, and March in 2011) from each tap outlet in sterile containers with 0.01% w/v sodium thiosulphate.

Three taps located in one patient room, the nurses' station and the doctor's office were installed with 0.2 μm POU filters (AQ14F1S, Pall Corp., Port Washington, NY, USA) for removal of the waterborne pathogens (Fig. 1). A pre-filtration fixture (pore size, 1.2 μm) was also installed for capturing particulate debris to extend the life of the POU filter. Filters were changed every 2 weeks according to the manufacturer's instructions from July to November 2010 (18 weeks), and water samples were collected and cultured every 3–4 days. The unfiltered tap water sample served as the control, while the water filtered through the pre-filter alone served as the pre-filtered water control. We picked the doctor's office and nurses' station for installation so that all medical staff had access to filtered (pathogen-free) water before and between patients' care.

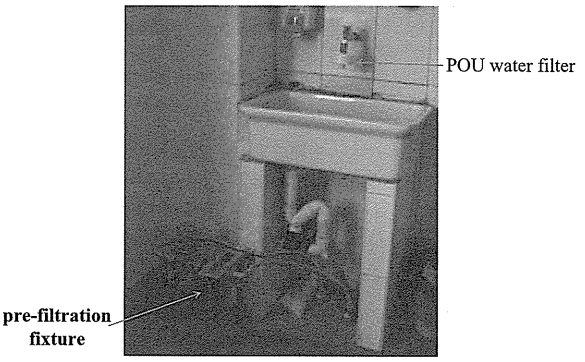

FIG. 1. Tap installed with POU water filter and pre-filtration fixture.

The incidence of Gram-negative bacteria colonization/infection in the LTU was also monitored. We analyzed patient-related data for the same 4-month period before the installation of the water filters (from July to November 2009) and a corresponding 4-month period after outlets had been equipped with filters (from July to November 2010). Patient data were retrieved from the hospital surveillance system. Microbiological cultures from patients were performed only when clinically indicated. No additional control measures were carried out during this period.

Microbiological analysis

Total heterotrophic plate count (HPC) bacteria, cultured on R_2A agar (Oxoid, Basingstoke, UK) at 25°C for 14 days, were enumerated by the standard pour plate method [12]. *Legionella* spp. was monitored using GVPC selected agar (Oxoid) according to ISO 11 731 [13]. Colonies morphologically consistent with *Legionella* spp. were identified by the latex agglutination test (Oxoid). For *Pseudomonas aeruginosa*, filamentous fungi and *Mycobacterium* spp. detection, water samples were filtered (pore size of 0.45 μm, Millipore, USA) and the filter membrane was placed on Cetrimide agar plates, Sabouraud dextrose agar plates containing 25 mg/L penicillin and 400 mg/L chloramphenicol (Oxoid) and Middlebrook 7H10 plates (BD, Franklin Lakes, NJ, USA), and incubated at 35°C for 48 h, 30°C for 28 days and 35°C for up to 8 weeks, respectively.

Statistical analyses

An ANOVA (SPSS ver. 15.0) was used to analyze the bacterial counts in POU-filtered, pre-filtered and unfiltered control samples. Comparison of the incidence of Gram-negative bacterium infection/colonization in the post-filtration period with that in the pre-filtration period was carried out by use of the chi-squared test (SPSS ver. 15.0). The correlation coefficient of temperature and the number of positive water samples were calculated by use of two-tailed Spearman's analysis.

Results

Baseline of waterborne pathogens in the LTU

A total of 136 cold water samples were enumerated for the targeted pathogens. *Legionella* spp, *Pseudomonas aeruginosa*, *Mycobacterium* spp. and filamentous fungi were detected in 38 (27.9%), 11 (8.1%), 118 (86.8%) and 68 (50.0%) water samples, respectively. HPC bacteria were detected in all the water samples, with a mean concentration of 1.1×10^7 CFU/L. Among the isolated *Legionella*, 29 of these (76.3% of positive samples and 21.3% of the total) were identified as *L. pneumophila*. Furthermore, 28.9% of *Legionella* spp.-positive samples were detected with high-level contamination ($\geq 10^3$ CFU/L). More than 18% (7/38) of the samples were positive for both *L. pneumophila* and *Legionella* of other species (Table 1).

Mycobacterium spp. was isolated from almost all water samples throughout the study. However, if we examine the data without considering *Mycobacterium* spp., sampling sites positive for target pathogens were higher in the hot season (from June to October), averaging 5.7 sites positive/month (40/7), compared with the cold season (from November to March), averaging three sites positive/month (9/3), which is almost a two-fold increase. Some pathogens seemed to persist in some outlets for a long time; for example, filamentous fungi were isolated from tap water of room one during the entire study period. We also found that the positive rate of *Legionella* spp. correlated with temperature fluctuations of tap water (correlation coefficient = 0.907; p 0.000), which suggested that cold water temperature below 20°C might be considered protective against *Legionella* contamination (Fig. 2).

Control modality using POU filter

As the water samples were found to be highly contaminated by *Legionella* spp. (10^3–10^4 CFU/L), three POU water filters were installed. From July to November 2010, a total of 190 water samples were collected from these three tap outlets, of which 57 were unfiltered water, 43 were pre-filtered water and 90 were POU-filtered water. No significant difference was observed in *Legionella* isolation between pre-filtered and unfiltered water (Table 2). In contrast, all samples filtered by the POU water filter were culture-negative for any of these pathogens. The difference in isolation between POU-filtered and control water was significant at $p < 0.05$. It is noteworthy that one of 34 (2.9%) water samples tested positive for HPC bacteria after 3 days use of filters, four of eight (42.1%) water samples tested positive after 7 days use, and the positive rate increased to 69.2% after 14 days use. Retrograde contamination may occur during use over time.

The number of Gram-negative bacterium infection/colonization patients per 1000 patient-days of hospitalization in the post-filtration period (1.70 ± 0.95) was significantly lower than that in the pre-filtration period (3.20 ± 1.25; $\chi^2 = 2.119$, p 0.067). Gram-negative bacterium colonizations/infections were reduced by 46.9%.

Discussion

Opportunistic waterborne pathogens can be introduced into a healthcare facility water distribution system. Despite water treatment and a chlorine disinfection process, treated water may still contain low concentrations of various microorganisms, such as *Legionella*, *P. aeruginosa*, non-tuberculous mycobacteria and fungi (e.g. *Aspergillus*). Pathogens can enter the water system of healthcare facilities and can colonize the water supply piping, hot water tanks, sinks, faucet aerators and shower heads. Hospital water distribution systems might be one of the most important sources of HAIs [1]. Thus, the World Health Organization (WHO) published its fourth edition of 'Guidelines for Drinking-Water Quality' [9], which specifically stated the importance of disinfection of the water supply as a control measure to prevent healthcare-associated infections. However, as in healthcare facilities throughout the world, no mandate exists for Chinese healthcare facilities to survey for waterborne pathogens in the water supply of healthcare facilities.

We conducted this prospective surveillance in the absence of any recognized outbreak attributable to waterborne pathogens of *Legionella* spp., *Pseudomonas aeruginosa*, non-tuberculous mycobacteria and filamentous fungi. A high prevalence rate of waterborne pathogens was found in the water supply of the

TABLE 1. Characteristics of pathogen contamination in the cold water samples without filter installation (n = 136)

Parameters	*Legionella* spp.	*Legionella pneumophila*	*Pseudomonas aeruginosa*	*Mycobacterium* spp.	*Filamentous fungi*	HPC bacteria[b]
Positive samples, No. (%)	38 (27.9)	29 (21.3)	11 (8.1)	118 (86.8)	68 (50.0)	136 (100.0)
Samples with $>10^3$ CFU/L, No. (%)	11 (8.1)	8 (5.9)	0	19 (13.9)	0	136 (100.0)
Geometric mean count (CFU/L [a], Mean (Range))	2.9×10^3 (50–5.8×10^4)	3.4×10^3 (100–2.0×10^4)	70.0 (5–3.6×10^2)	5.9×10^2 (2–5.0×10^3)	41.5 (10–62)	1.1×10^7 (1.0×10^4 –3.4×10^8)

[a]Only positive samples were included.
[b]HPC, heterotrophic plate count.

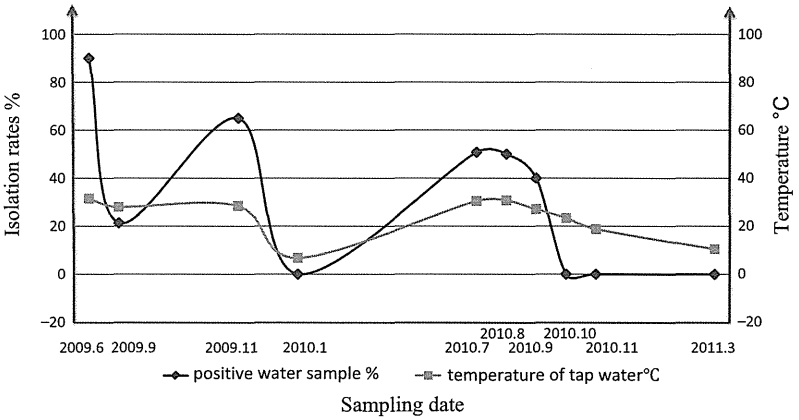

FIG. 2. Relationship between contamination rate of tap water and temperature (*Legionella* spp.).

TABLE 2. Growth of different pathogens and HPC bacteria in unfiltered, pre-filtered and POU-filtered water samples

			Mean concentration of organisms in positive samples (CFU/L)			
	Total No. of samples	**No. of positive samples (%)**	**HPC bacteria**	**Legionella**	**Mycobacterium**	**Filamentous fungi**
Unfiltered water	57	57 (100)	1.2×10^8	1.0×10^3	4.0×10^2	16.5
Pre-filtered water	43	43 (100)	3.7×10^8	1.8×10^3	7.0×10^2	12.7
Filtered water	90	34 (37.7)	3.4×10^4	0	0	0
Filtered water after 3-day interval	34	1 (2.9)	0	0	0	0
Filtered water after 7-day interval	19	8 (42.1)	1.3×10^4	0	0	0
Filtered water after 10-day interval	21	7 (33.3)	5.4×10^4	0	0	0
Filtered water after 14-day interval	26	18 (69.2)	2.6×10^4	0	0	0

LTU. During the interventional control strategy using POU water filter, we found that POU filters completely eliminated these waterborne pathogens from the water supply over 14 days of use. Furthermore, the rate of Gram-negative bacterium infection/colonization patients per 1000 patient-days of hospitalization in the post-filtration period (1.70 ± 0.95) decreased significantly compared with the pre-filtration period (3.20 ± 1.25; $\chi^2 = 2.119$, $p = 0.067$), a 47% reduction! Our study demonstrated that POU water filters provided a barrier against various waterborne pathogens that can further reduce the rate of nosocomial infections. However, the retrograde contaminations may occur by either splash water from the water basin during use or by direct contact with contaminated hands and dirty clothes of staff or patients [14,15]. In our surveillance, HPC bacteria were recovered from the filtered water after 1 week of use. The source of the HPC bacteria from filtered water remains unclear, and molecular typing may be useful to track the dissemination.

Due to the poor quality of the supplied tap water in the LTU, we installed pre-filtration fixtures on three taps upstream of POU filters in order to remove particulate debris. Before the POU filter study, laboratory and field tests were conducted for evaluating the performance of pre-filtration of various materials and styles and removal ratings, and 1.2-μm pore size was chosen for the pre-filtration fixture (data not shown). The pre-filtration fixture would not prohibit the waterborne pathogens from tap water. However, the concentration of various pathogens after pre-filtration was surprisingly higher than that in unfiltered water in some samples. The reason may be the growth of pathogens within the pre-filtration media because of higher nutrient content from the trapped debris in the water.

Although only 27% of distal sites (3/11) were equipped with POU filters, the incidence of Gram-negative bacterium colonization/infection decreased significantly, possibly because of the use of filtered tap water for perineal washing of patients, the bed environment and the hands of nursing personnel. In this study, it remains unclear whether such filters contribute to the reduction of non-tuberculosis mycobacterium and filamentous fungi infections in high-risk patients. Therefore, more research is needed to evaluate the efficacy and cost-effectiveness of POU filters in preventing specific colonization/infection of hospitalized patients. Many studies have focused primarily upon recognized outbreaks of *Legionella* spp. and *P. aeruginosa* [16,17]. However, other opportunistic waterborne pathogens may also cause nosocomial infections, outbreaks or sporadic infections [18,19]. There is controversy over whether it is economical to invest medical resources in preventing opportunistic waterborne pathogen-associated nosocomial infections, especially because the disposable POU water filters have a limited effective life and could be very expensive. We propose a modest approach whereby removal of waterborne

pathogens is targeted towards areas of highest risk of nosocomial infections, such as our transplant unit. In such settings the costs are likely to be justifiable. We should remove waterborne pathogens from transplantation units because these patients are at the highest risk of nosocomial. If an organ transplant patient dies from nosocomial infection, a valuable organ is also being destroyed. Given such a high potential cost associated with nosocomial infections in transplant patients, POU water filters may be a viable economical option [20,21]. Instead of treating the entire hospital water supply with systematic chemical disinfection (e.g. chlorination), POU filters can be easily installed at a few sites for prevention of infection [22–24]. Furthermore, based on our data, we suggest installing POU water filters only in the hot season (June to October) in countries with limited medical resources.

In conclusion, hospital water supplies were highly contaminated by various waterborne pathogens. Using POU filters appeared to be one of the most simple and cost-effective methods to reduce the risk of waterborne pathogen-associated infections in hospitals.

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Transparency Declaration

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Original article

The relationship between biofilm formations and capsule in *Haemophilus influenzae*☆

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ABSTRACT

To evaluate the biofilm formation of non-typeable *Haemophilus influenzae* (NTHi) and *H. influenzae* type b (Hib) clinical isolates, we conducted the following study. Serotyping and polymerase chain reaction were performed to identify β-lactamase-negative ampicillin (ABPC)-susceptible (BLNAS), β-lactamase-negative ABPC-resistant (BLNAR), TEM-1 type β-lactamase-producing ABPC-resistant (BLPAR)-NTHi, and Hib. Biofilm formation was investigated by microtiter biofilm assay, as well as visually observation with a scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) in a continuous-flow chamber. As a result, totally 99 strains were investigated, and were classified into 4 groups which were 26 gBLNAS, 22 gBLNAR, 28 gBLPAR-NTHi and 23 Hib strains. The mean OD_{600} in the microtiter biofilm assay of gBLNAS, gBLNAR, gBLPAR-NTHi, and Hib strains were 0.57, 0.50, 0.34, and 0.08, respectively. NTHi strains were similar in terms of biofilm formations, which were observed by SEM and CLSM. Five Hib strains with the alternated type b *cap* loci showed significantly increased biofilm production than the other Hib strains. In conclusion, gBLNAS, gBLNAR, and gBLPAR-NTHi strains were more capable to produce biofilms compared to Hib strains. Our data suggested that resistant status may not be a key factor but capsule seemed to play an important role in *H. influenzae* biofilm formation.

1. Introduction

Haemophilus influenzae is a pleomorphic gram-negative rod-shaped bacterium that colonizes the human nasopharynx. Non-typeable *H. influenzae* (NTHi) can cause a variety of infections, including otitis media, sinusitis, conjunctivitis, bronchitis, and pneumonia [1], whereas *H. influenzae* type b (Hib) is reported as a common cause of meningitis and other invasive infections especially in children [2]. As well known, biofilms are defined as communities of microorganisms that attach to a surface and are enveloped in a hydrated polymeric matrix of their own synthesis [3]. It has recently been reported that NTHi is able to form biofilms in vitro [4] and on middle-ear mucosa in the chinchilla model of otitis media [5]. However, there is still very few information about biofilm formations in Hib at present. Furthermore, it has also recently been reported that β-lactamase-negative ABPC-resistant (BLNAR) *H. influenzae* have increased in some countries [6,7], although their global prevalence remains low [8]. Since biofilm-producing *H. influenzae* were recognized as a new issue in treatment, investigations of biofilm formation of antibiotics resistant strains such as BLNAR or β-lactamase-producing ABPC-resistant (BLPAR) became important. The aim of this study was to evaluate the biofilm formations of clinical isolates with different characteristics of antimicrobial susceptibility.

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2. Methods

2.1. Bacterial strains

All strains were stored at −70 °C. Strains were inoculated on chocolate agar plates and cultured at 37 °C overnight in 5% CO_2 for subculture. All strains were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, Mich, USA). Twenty-three type b strains were investigated using conventional polymerase chain reaction (PCR) with the capsule type b primers (b1–b2) that amplified capsule type-specific DNA fragments (480 bp). The sequences specific for Hib were 5′-GCGAAAGTGAACTCTTATCTCTC-3′ and 5′-GCTTACGCTTCTATCTCGGTGAA-3′ [9]. Region III–I primers (5′-GTGATTATTGATCTGCCCCCTAAAAGTTGGACAGGTTAG-3′ (15507–15545) and 5′-GGGGATAACATGACAACCG AAAATCCGGCAATACCGACG-3′ (20504–20542), GeneBank: AF5492 13) was designed for amplifying the partial *H. influenzae cap* loci including IS*1016*, *bexA*, *bexB*, and *bexC* genes, according to the nucleotide sequences described previously [10–12]. β-lactamase production was detected by means of a disc impregnated with nitrocefin (Becton Dickinson, Sparks, MD, USA).

2.2. Antimicrobial susceptibility test

MICs were determined by the agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute [13]. The susceptibilities of all the strains against the following 6 antibiotics were tested: Ampicillin (ABPC) (Meiji Seika Kaisha, Tokyo, Japan), Ampicillin/sulbactam (ABPC/SBT) (Pfizer Japan Inc, Tokyo), Cefotaxime (CTX) (Aventis Pharma, Tokyo), Cefotiam (CTM) (Takeda Chemical Industries, Osaka, Japan), Levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Tokyo) and Meropenem (MEPM) (Sumitomo Chemical Co., Tokyo).

2.3. PCR for identification of resistance genes

PCR for the identification of resistance genes was carried out for *H. influenzae* isolates using mixed primers (Wakunaga Pharmaceutical Co., Hiroshima, Japan), as described previously [14]. Briefly, P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae* (198 bp); TEM-I primers to amplify a part of the bla_{TEM-1} gene (458 bp); PBP3-S primers to identify an Asn526→Lys amino acid substitution in the *ftsI* gene (551 bp); and PBP3-BLN primers to identify an Asn526→Lys and Ser385→Thr amino acid substitution in the *ftsI* gene (465 bp). gBLNAS, gBLNAR and gBLPAR were determined followed the manufacturer's protocol.

2.4. Microtiter biofilm assay

Biofilm productions were investigated using a microtiter biofilm assay (MBA), as described previously [4]. Each strain was cultured for overnight in BHI and bacteria solution was diluted in 1:200 with fresh broth. 200 μl aliquots were inoculated into the wells of a 96 well micro-plate (Nalge Nunc International Co. Naperville, IL), and were incubated at 37C°, 5% CO_2 for 24 h. Before biofilm quantitation, bacteria growth was measured by using a micro-plate reader at OD_{490}. Twenty μl of freshly adjusted crystal violet (Fisher Scientific, Pittsburgh, PA) was added to each well and incubated at room temperature for 15 min. The plates were then washed vigorously with distilled water and dried. A volume of 230 μl of 95% ethanol was added to each well, and then the biofilm formation was measured at OD_{600}. All strains were tested in quadruplicate and the results were reported as average ± SD of three different experiments.

2.5. Visually observation of biofilms

2.5.1. SEM

The biofilm architectures were observed by scanning electron microscopy (SEM) [15]. In a typical experiment, coverslips were fixed in a 2% osmium 0.1 M Na cacodylate buffer for 1.5 h, dehydrated with ethanol washes, and dried with a critical point dryer to preserve the biofilm structure. The processed coverslips were then mounted on stubs using colloidal silver and were sputter coated with gold palladium.

2.5.2. Biofilm growth in a continuous-flow chamber

A continuous-flow chamber was used for biofilm formation as described previously [16]. 1 ml of BHI broth containing *H. influenzae* (1×10^8 cfu/ml) was inoculated into the chamber, and incubated for 1 h. BHI media diluted 1:10 with PBS (at a flow rate of 199.5 μl/min) was then added. Biofilms were allowed to form in a continuous-flow chamber for 2 days, and then observed by confocal laser scanning microscopy (CLSM), as previous study described [17]. In a brief, the flow chamber was carefully disconnected, biofilms formed on the surface of the glass coverslip of the chamber were stained with SYTO 9 and propidium iodide which were mixed at a 1:1 ratio (Molecular Probes, Eugene, Oreg), and incubated for 15 min at 37 °C. Biofilms were observed with a Bio-Rad MRC-1024 laser scanning confocal viewing system at a magnification of ×20. The resulting images were complied as cross sections of a z series.

3. Results

3.1. Characteristics of H. influenzae isolates

Twenty-six gBLNAS-NTHi strains were isolated from the nasopharynx (13 strains) and sputum (13 strains), 22 gBLNAR-NTHi were isolated from the nasopharynx (16 strains), middle ear (3 strains) and conjunctiva exudate of the eye (3 strains), 28 gBLPAR-NTHi strains were isolated from the nasopharynx (16 strains) and sputum (12 strains), and 23 Hib strains were isolated from the cerebrospinal fluid (20 strains) and nasopharynx (3 strains). Of the 23 Hib strains previously determined by slide agglutination assays, 18 (No. 5–19, 21–23) possessed the capsule type-specific DNA fragments (480 bp), and the partial Hib *cap* loci (5036 bp). While no targeted DNA fragments were detected in the remaining 5 strains (No. 1–4, 20), which suggested that mutations might have occurred in both investigated regions (Fig. 1A, B).

3.2. Antimicrobial susceptibility test

Table 1 showed the antimicrobial susceptibilities of all the strains against the 6 tested antibiotics against 26 gBLNAS, 22 gBLNAR, 28 gBLPAR-NTHi, and 23 Hib strains. The MICs of gBLNAR-NTH against ABPC, ABPC/SBT, CTX, CTM and MEPM were more resistant than those of gBLNAS, gBLPAR-NTHi and Hib. Whereas MICs against LVFX were low and similar among these 4 groups of *H. influenzae*.

3.3. Biofilm formation

The results of MBA were represented with the mean OD_{600} of gBLNAS, gBLNAR, gBLPAR-NTHi, and Hib strains as following 0.57 (range: 0.01–1.48), 0.50 (range: 0.13–1.19), 0.34 (range: 0.01–0.96), and 0.08 (range: 0–0.86), respectively. Variable abilities of biofilm production among gBLNAR, gBLNAS and gBLPAR-NTHi strains were detected (Fig. 2). Most Hib strains did not seem to produce biofilms, but 5 Hib strains showed significantly higher OD_{600} compared to those of the remaining Hib strains ($p = 0.0045$, Mann–Whitney's U

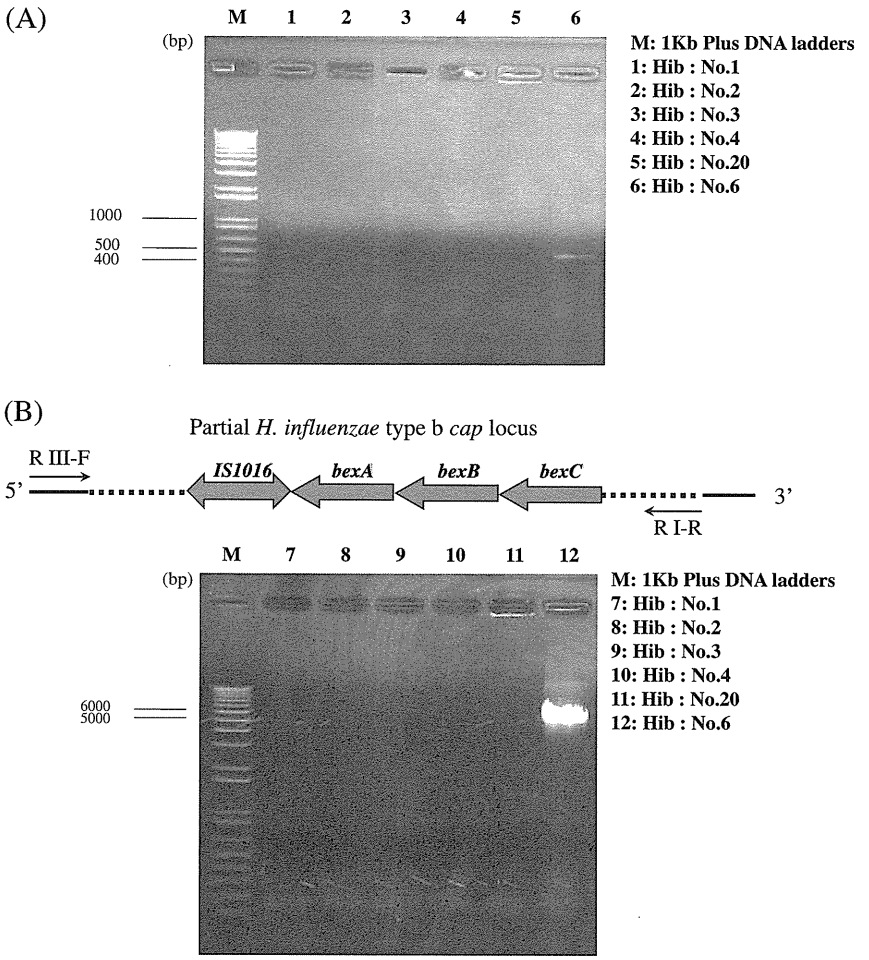

Fig. 1. A. Investigation of capsule type-specific DNA fragments. Capsule type b primers (b1–b2) were designed for confirmation of capsule type-specific DNA fragments (480 bp). B. Investigation of *H. influenzae* type b *cap* loci. Region III–I primers were designed to span a partial *H. influenzae* cap loci including IS*1016*, *bexA*, *bexB*, and *bexC* genes (5036 bp).

test). These 5 strains were confirmed with alternated Hib *cap* loci, 3 of which (No.1–3) were isolated from nasopharynx, and the remaining strains (No.4, No.20) were from cerebrospinal fluid.

NTHi strains were able to form mature biofilms with similar architectures, which were observed by SEM. On the other hand, the Hib strain (No. 6) did not produce biofilms with a weak attachment to the surfaces, but the No.3 strain formed a mature biofilm, which was confirmed with alternated type b *cap* loci (Fig. 3A). The same results have also been detected in a continuous-flow chamber with CLSM (Fig. 3B).

4. Discussion

Biofilm formation and the inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections [3]. Otitis media caused by NTHi constitutes one of such infections [3,5]. The emergence of drug-resistant NTHi such as the BLNAR strains has dramatically changed its clinical outcome in some countries [6,7], and not only antibiotics but also myringotomy is occasionally required as treatment. Although it has been reported that biofilm-producing *Pseudomonas aeruginosa* tend to be more resistant to antibiotics [18,19], there is still few information about the differences of biofilm formations between susceptible and resistant isolates such as BLNAR or BLPAR in *H. influenzae*. Our results demonstrate the capability of forming biofilms by resistant NTHi is similar to that by susceptible isolates overall, and which is different from *P. aeruginosa*. Furthermore, we found 9 (40.9%) and 14 (53.8%) strains in gBLNAR and gBLNAS-NTHi produced biofilms with OD_{600} value higher than 0.5, respectively, compared to 8 (28.6%) strains in gBLPAR-NTHi. These results suggested that β-lactamase might involve in *H. influenzae* biofilm formation. There is not any evidence to support this hypothesis in the present study, so we suggest future study should focus on this aspect.

The issue that whether Hib, the most common cause of bacterial meningitis in children, is capable to produce biofilm is still unclear. Previous studies demonstrated that biofilm matrix seemed to be regulated by CPS, protein components, and other extracellular molecules, and the rate at which bacteria attach to a surface is an important determinant of biofilm formation [20,21]. Our results demonstrated that most clinical Hib strains produced lesser amounts of biofilms compared to NTHi. Interestingly, we detected 5 Hib strains produced biofilms, and which were confirmed with alternated type b *cap* loci. As well known, all encapsulated *H. influenzae* strains contain functionally unique regions I, II, and III for the production of their respective polysaccharide capsules found within the *cap* loci, which is flanked by direct repeats of insertion element *IS1016* and is frequently amplified. Regions I (bexDCBA) and III are common to all six capsular types and contain

Table 1
Antimicrobial susceptibilities of *H. influenzae* strains.

Antibiotics	Resistant class	MICs (μg/ml)		
		Range	50%	90%
Ampicillin	gBLNAS-NTHi	0.063–0.5	0.25	0.5
	gBLNAR-NTHi	1–8	2	8
	gBLPAR-NTHi	1–64	16	32
	Hib	0.125–64	1	8
Ampicillin/sulbactam	gBLNAS-NTHi	0.032–0.5	0.125	0.25
	gBLNAR-NTHi	0.5–2	1	1
	gBLPAR-NTHi	0.25–1	0.5	0.5
	Hib	0.032–2	0.25	0.25
Cefotaxime	gBLNAS-NTHi	0.008–0.063	0.016	0.032
	gBLNAR-NTHi	0.063–2	1	2
	gBLPAR-NTHi	0.016–0.063	0.016	0.063
	Hib	0.004–0.063	0.016	0.032
Cefotiam	gBLNAS-NTHi	0.5–1	1	1
	gBLNAR-NTHi	2–≧128	64	≧128
	gBLPAR-NTHi	0.5–2	1	2
	Hib	0.063–8	1	1
Levofloxacin	gBLNAS-NTHi	0.016–0.063	0.032	0.063
	gBLNAR-NTHi	0.008–0.063	0.032	0.063
	gBLPAR-NTHi	0.016–0.063	0.032	0.063
	Hib	0.008–0.063	0.032	0.063
Meropenem	gBLNAS-NTHi	0.016–0.125	0.063	0.063
	gBLNAR-NTHi	0.125–0.5	0.5	0.5
	gBLPAR-NTHi	0.063–0.25	0.063	0.125
	Hib	0.016–0.25	0.063	0.125

BLNAS: β-lactamase nonproducing-ampicillin susceptible *H. influenzae*; BLNAR: β-lactamase nonproducing-ampicillin resistant *H. influenzae*; BLPAR: β-lactamase producing-ampicillin resistant *H. influenzae*.

genes necessary for the processing and exportation of the capsular material, while region II is capsule type specific [11,12]. Thus we hypothesized that alternated type b *cap* loci might interrupt the expression of capsule and result in loosing the innate b capsule, and which might facilitate colonization and biofilm formation in these strains. Previous studies also reported that the frequency of spontaneous capsule loss in Hib is 0.1–0.3% [22], and capsule loss in Hib results in enhanced adherence to and invasion of human cells [23]. Although *H. influenzae* recovered from the respiratory tract generally showed non-encapsulated (serologically non-typeable), whereas isolates from systemic sites typically expressed encapsulated, especially a b capsule, some NTHi isolates from pharynx were proven to originate from an encapsulated ancestor [24].

Unfortunately, we could not investigate the capsule variation at a protein level, as well as the relatedness between biofilm formation and capsule, future work should focus on these aspects. It has also been reported that the expression of capsule polysaccharide (CPS) in *Vibrio vulnificus* inhibits biofilm formation, and impaired pneumococcal CPS may increase biofilm formation and involve in the inhibition of the virulence possibly due to influencing the gene expression in *Streptococcus pneumoniae* [20,25]. The mechanism for why the loss of b capsule in Hib leads to enhance biofilm formation seemed to be complicate. Pili has been reported as an important structure for adherence [4], and biofilm formations [26,27] in *H. influenzae*. Since most Hib strains seemed not to produce biofilms, we thus speculate that a capsule might shield the function of the pili in the prior attachment was responsible for impaired biofilm formation.

On the other hand, genes involved in capsule expression are major virulence factors of *H. influenzae* [11,28]. It has been recently reported that a *H. influenzae* type a strain isolated from cerebrospinal fluid of a child with meningitis and a non-encapsulated (serologically non-typeable) *H. influenzae* strain simultaneously obtained from blood of the same patient showed the similar genetic structure for the cap region [10]. These data suggested that there is a possibility that Hib strains even lose their capsule and colonize in the nasopharynx, which may still have sufficient virulence to cause an invasive disease in human, and which is a high concern for clinicians.

Our data indicate that *H. influenzae* may produce mature biofilms without regard to their resistant status. Hib strains with alternated type b *cap* loci produced biofilms might be due to interruption of the b capsule expression. Therefore, future research conducting the relatedness of the loss of capsule, biofilm formation and virulent factors should be necessary.

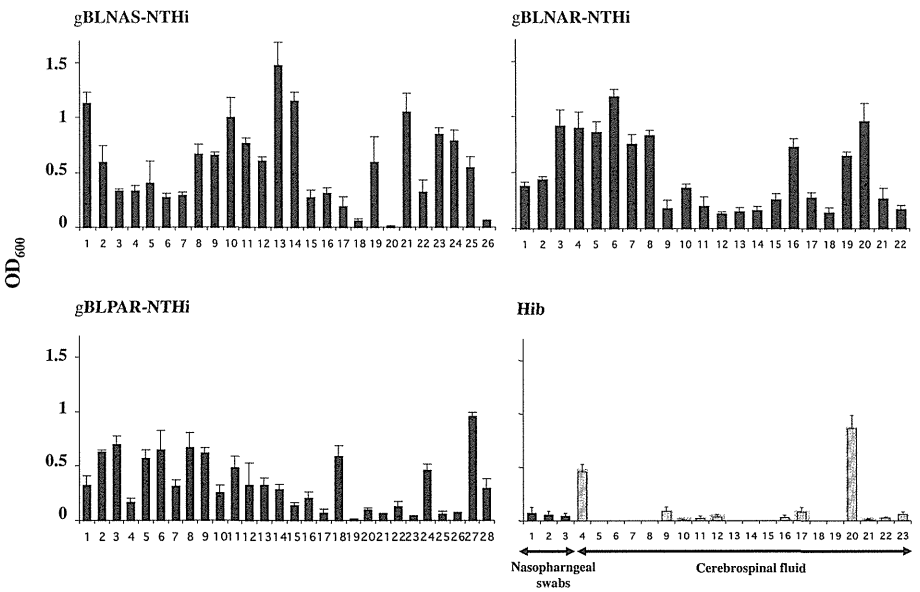

Fig. 2. Biofilm formation in each resistant class of *H. influenzae* strains. BLNAS: β-lactamase nonproducing-ampicillin susceptible *H. influenzae*; BLNAR: β-lactamase nonproducing-ampicillin resistant *H. influenzae*; BLPAR: β-lactamase producing-ampicillin resistant *H. influenzae*. All strains were tested in quadruplicate and the results were reported as average ± SD of three different experiments.

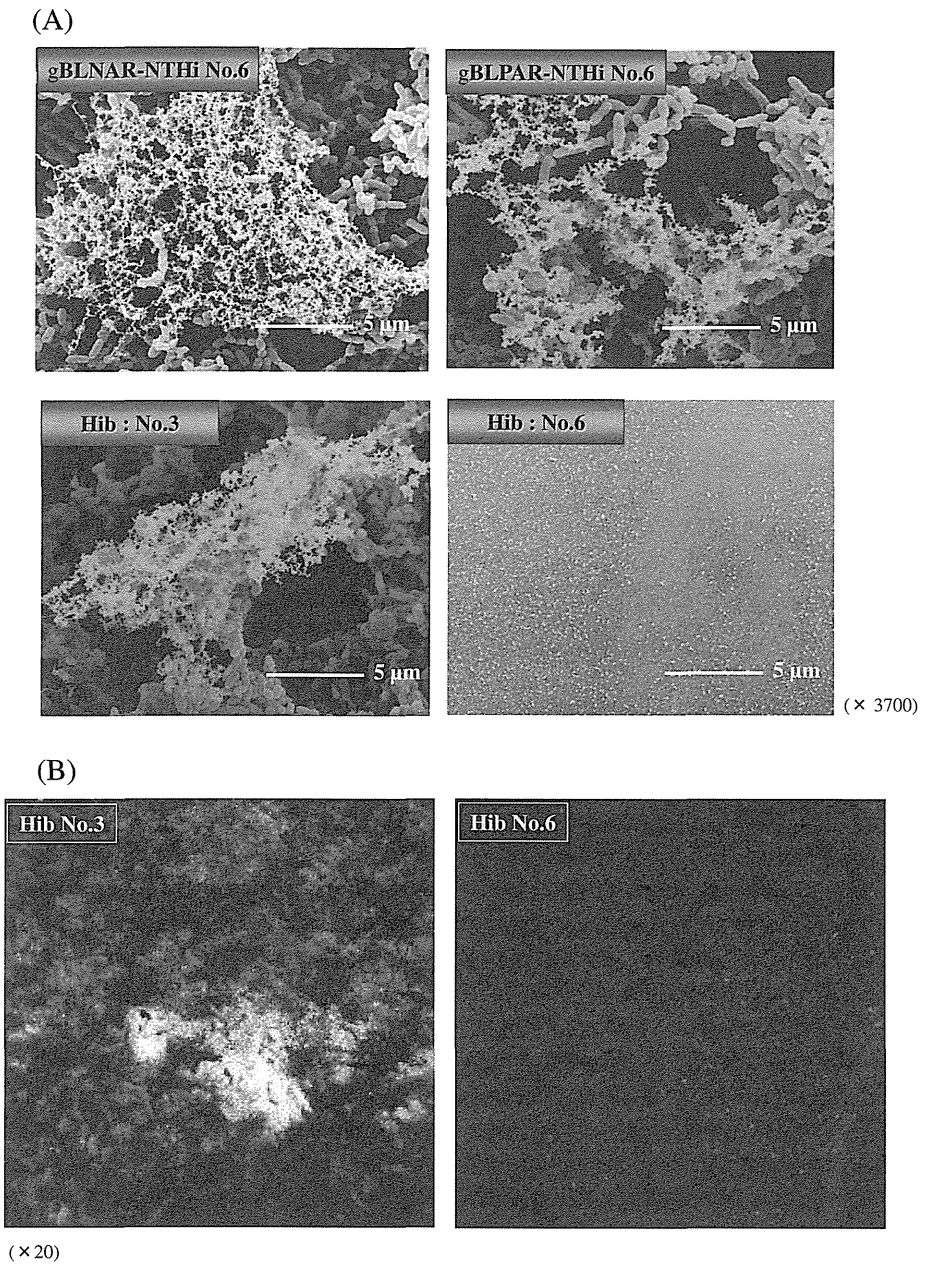

Fig. 3. A. Biofilm architectures observed by SEM. SEM showed that gBLNAR-NTHi (No. 6), gBLPAR-NTHi (No. 6), and Hib strain (No. 3) clearly produced mature biofilms, whereas Hib strain (No. 6) did not produce a biofilm. B. Biofilm architectures in a continuous-flow chamber observed by CLSM. Biofilms grown in the chamber were observed with a Bio-Rad MRC-1024 laser scanning confocal viewing system at a magnification of ×20. The resulting images were complied as cross sections of a z series. CLSM showed the Hib strain (No. 3) was able to form a biofilm, but the Hib strain (No. 6) failed to produce a biofilm.

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The authors declare that they have no conflict of interest.

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Performance of a rapid human metapneumovirus antigen test during an outbreak in a long-term care facility

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SUMMARY

Using a newly developed rapid test, an outbreak of human metapneumovirus (HMPV) infection in a long-term care facility was detected within only 2 days after the onset of symptoms in a putative index case. The outbreak was almost under control within 8 days mainly by zoning patients, with the exception of two cases of HMPV that were diagnosed 16 and 17 days after the onset of the outbreak. According to an immunological diagnosis as well as the rapid test, it was eventually proven that 18 patients had HMPV infections. We suspected that even asymptomatic residents, who had not been completely separated from the facility population, were a source of infection. That suggested that all asymptomatic residents should be tested and that the separation of the infected patients should be absolute, if an outbreak of HMPV infection is suspected in such a facility.

Key words: Human metapneumovirus, long-term care facility, rapid HMPV antigen test.

INTRODUCTION

Human metapneumovirus (HMPV) is an important pathogen of the lower respiratory tract that most often affects hospitalized children, immunocompromised patients [1] and elderly occupants of long-term care facilities [2, 3]. Due to the close proximity of living quarters and the reduced levels of personal hygiene and movement of the residents, outbreaks of fatal influenza or norovirus infections have been reported in such facilities [4, 5]. A rapid test has become a very useful tool in preventing the spread of infections because it results in rapid identification and isolation of the index case. A rapid HMPV antigen test has not been available until recently and the sensitivity and specificity of the assay have been described [6]. The present study focused on evaluating the performance of this new HMPV rapid test during a recent outbreak of HMPV infection in a long-term care facility.

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METHODS

Nasopharyngeal swab specimens were tested using both the Check hMPV assay (Meiji Seika Pharma Co. Ltd, Japan) and a nested RT–PCR [7]. The amplicon fragment (357 bp) was extracted using

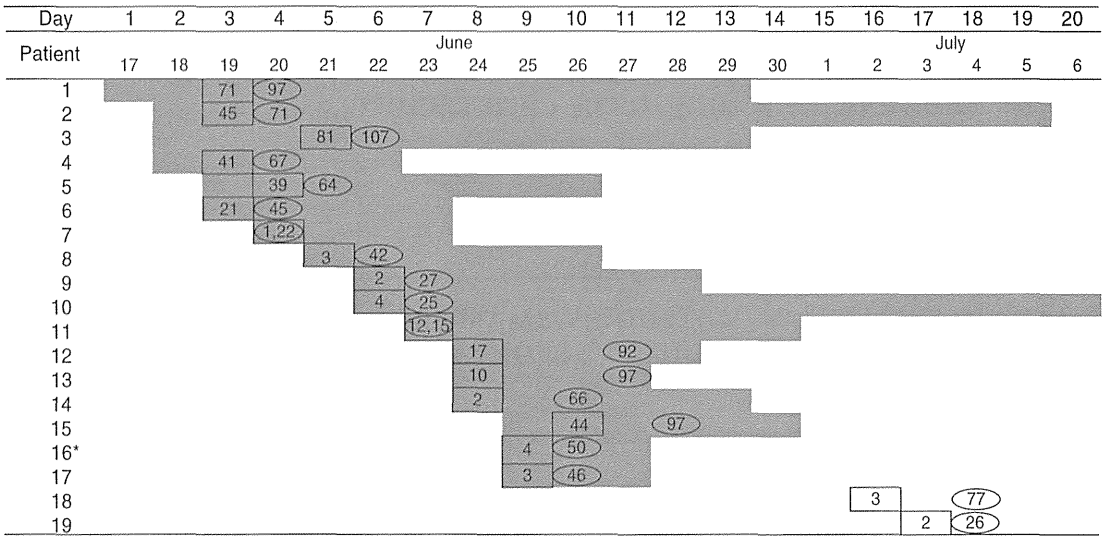

Fig. 1. The time-course of the outbreak of human metapneumovirus (HMPV) infection. The grey shading indicates the duration of fever (body temperature >37·5 °C); rectangles indicate the day of diagnosis by the rapid test (numbers within rectangles represent hours after onset of symptoms); ellipses indicate the day of collection of the acute serum and nasopharyngeal swab (for HMPV RNA extraction) (numbers within ellipses represent hours after the onset of symptoms). * Patient 16 was accommodated in an intensive respiratory care unit on 28 June.

GeneCleanII (MP Biomedicals, USA). The sequence was determined using a primer specific for the HMPV F gene (5′-CATGCCGACCTCTGCAGGAC-3′, 5′-ATGTTGCAYTCYYTTGATTG-3′) (FASMAC, Kanagawa, Japan). This rapid test was previously known as the SAS hMPV test [6]. An indirect fluorescent antibody test for measuring IgM and IgG antibodies was performed to confirm the HMPV infection as described previously [2, 8]. The serological test was performed once on the serum of all patients. In addition to the detection of viral antigens and nucleic acids, a fourfold increase in serum HMPV IgG titre 2 weeks after onset of symptoms provided evidence of positive immune responses to HMPV. For virus isolation, nasopharyngeal swab samples were suspended in Eagle's minimum essential medium (MEM), and the suspension was inoculated onto LLC-MK2 and Vero E6 cells. The cells were cultured in MEM containing trypsin (1 μg/ml). Isolation of HMPV was confirmed via RT–PCR and the rapid test. The present study (number 09040) was approved by the Ethics Committee of Kurume University.

RESULTS

A total of 18 patients were affected with HMPV over a 20-day period during June–July 2011, in a long-term care facility in Kurume, Japan (Fig. 1). One of 45 residents (case 6) on the first floor and 17 of 41 residents on the second floor were affected in one of several building units in this facility. All residents of each floor were accommodated in one open space. Most patients had a fever lasting 3–6 days. There were 1–3 new patients diagnosed daily during the first 8 days. Using the rapid test, we detected the outbreak in only 2 days after the onset of symptoms in the putative index case (case 1). Infection control measures were instituted to prevent the further spread of infection, which included zoning patients and hand washing. The zone where patients resided was surrounded by a long vinyl curtain in a corner of the ward. Additional patients continued to be diagnosed over the 4- to 5-day HMPV infection incubation period [9]. However, the outbreak was almost under control within only 8 days. Two patients were diagnosed 16 and 17 days after the onset of the outbreak. Case 16 was admitted to the intensive-care unit of another hospital with severe respiratory failure. The attack rates on the first and second floors were 2·2% and 44%, respectively. The total attack rate was 19·8%, which was smaller than that in previous reports [2, 10]. There was no record concerning HMPV infection in staff members.

According to an immunological diagnosis, as well as to the rapid test, it was eventually proven that all symptomatic patients, except for case 11, had been

Table 1. *Clinical features of symptomatic patients during a human metapneumovirus (HMPV) outbreak*

Patient no.	Age (yr)	Sex	WBC (/mm^3)	CRP (mg/dl)	Rapid test*	RT–PCR†	Virus‡	IgM titre			IgG titre			
								Acute	2 wk	1 yr§	Acute	2 wk	Ratio	1 yr§
1	32	M	7000	5	+	+	–	20	80	<10	160	2560	16	320
2	28	M	4400	3	+	+	–	<10	10	<10	80	2560	32	80
3	6	F	3700	2	+	+	–	160	320	20	320	10240	32	640
4	17	M	7000	2	+	+	–	40	40	<10	80	10240	128	160
5	17	M	9400	0	+	+	–	1280	160	40	40	5120	128	40
6	42	F	7600	6	–	–	–	80	40	80	10	320	32	640
7	13	M	5200	0	–	–	–	640	80	10	1280	5120	4	320
8	41	M	4600	2	+	+	–	160	320	<10	640	5120	8	160
9	21	M	7400	12	+	+	+	320	160	<10	640	2560	4	160
10	42	F	7700	2	+	+	–	40	160	<10	1280	10240	8	320
11	40	M	5300	2	–	–	–	320	<10	<10	1280	2560	2	80
12	27	M	12000	1	+	+	–	10	80	80	1280	2560	2	160
13	36	F	6800	4	+	+	–	80	80	<10	40	5120	128	80
14	43	M	5400	6	+	+	–	40	80	<10	320	5120	16	160
15	4	F	8400	4	+	+	+	320	160	<10	640	5120	8	80
16	26	M	9000	5	+	–	–	160	320	<10	2560	5120	2	80
17	41	F	6200	1	+	+	–	320	320	<10	40	1280	32	160
18	3	F	8200	0	+	+	+	640	1280	<10	160	1280	8	80
19	10 mo.	F	11100	<0·2	+	+	–	320	160	40	20	160	8	160

WBC, White blood cells; CRP, C-reactive protein.
* Check hMPV assay (Meiji Seika Pharma Co. Ltd, Japan).
† Genbank accession numbers of the amplicon (F gene) are JX966477–JX966485.
‡ Virus isolation usingLLC-MK2 cells.
§ IgM and IgG titres were evaluated 1-year post-outbreak of HMPV.

infected with HMPV (Table 1). Of these, 16 (84%) patients were positive for HMPV according to the rapid test result. These results were confirmed using a nested RT–PCR, with the exception of case 16. HMPV was isolated in the samples from cases 9, 15 and 18. Cytopathic effects of LLC-MK2 cells were seen and rounded and fused cells were clearly observed. The sequences of the amplicon, which codes for a part of the HMPV F protein, were completely identical in all sequences obtained (GeneBank accession numbers JX966477–JX966485), which suggested that the outbreak of HMPV infection would have originated from an index case.

Figure 1 shows the duration of fever and the time lag-between the onset of symptoms and the diagnosis (rapid test, RT–PCR, immunological test). The time lag of the rapid test in nine cases was around 1–4 h. Of these nine cases, only case 7 was negative by the rapid test. The time lag for the remaining cases was between 10 and 81 h. Of these cases, only two were found to be negative by the rapid test. The time lag was not related to the detection rate. Based on the RT–PCR results, the negative results were evenly detected in several cases. The time lag between the onset of symptoms and the collection of the nasopharyngeal swab for RNA extraction varied from short to long.

DISCUSSION

In this study, we were able to detect an outbreak of HMPV infection in a long-term care facility at an early phase using a new rapid test. Early detection of HMPV enabled us to prevent widespread infection at the facility and the outbreak had almost ceased within 8 days. We were also able to keep the affected number of patients to within 17 out of a total of 41 residents in a ward on the second floor and to 1 out of 45 in another ward on the first floor.

Influenza outbreaks have been reported in a long-term care facility and fatal cases are common. Many patients in this report had a high fever for at least 3–7 days (Fig. 1). This period seemed to be longer than that seen for influenza infections. However, fatal cases of HMPV infection are relatively rare [2, 11]. Early detection of the HMPV outbreak increased the prospects for a good outcome.

In total, 16 (89%) of the 18 HMPV patients were found to be positive using the rapid test. The rapid

test and RT–PCR results were in accord except for case 16. The time lag between the onset of symptoms and the collection of a nasopharyngeal swab for RT–PCR was 50 h, but the time lag between the onset of symptoms and the rapid test was only 4 h. That suggested that the amount of HMPV might have decreased by the time the nasopharyngeal samples were collected. With respect to the rapid test-positive results for cases 12 and 16, no significant increase in serum IgG titre was detected 2 weeks after the onset of symptoms, because the titre of IgG in acute serum had already increased. This might suggest that cases 12 and 16 were asymptomatically infected with HMPV at a much earlier time, although the time lag between the onset of symptoms and the collection of serum samples was relatively early (within 3–4 days). The role of asymptomatic patients in comparatively large HMPV outbreaks has been reported previously [12].

False-negative rapid test results were noted for cases 6 and 7. These patients were also immediately isolated in the same separated area as the rapid test-positive patients because they showed similar clinical symptoms. It is important to note that cases 6 and 7 eventually tested positive for HMPV according to the serological test. This result suggested that the isolation of all symptomatic patients, including patients with false-negative results, was an appropriate control measure to prevent the spread of infection.

We concluded that the rapid HMPV test had high sensitivity, making it very useful for the early detection of an outbreak of HMPV infection in a long-term care facility. Our study also suggested that even asymptomatic patients might be a source of infection. Therefore, we propose rapid diagnosis testing for all asymptomatic residents as well as for symptomatic patients. Testing the asymptomatic residents might be an important measure in preventing the emergence of secondary-infected patients in such a long-term care facility.

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DECLARATION OF INTEREST

None.

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トラベラーズワクチン

渡邊　浩*

要旨

・近年，わが国の海外渡航者数は増加し，渡航先や形態にも変化がみられ，海外渡航者がさまざまな感染症に罹患する危険性が増している。
・わが国においては都市部ではトラベルクリニックは増えているものの地方ではまだ少なく，地域によっては海外渡航時のワクチンを接種できる医療機関がほとんどないという場合も珍しくない。
・本来，海外渡航をする人は渡航地の感染症情報や治安状況を事前に調べ，ワクチン接種をはじめとする必要な感染症予防対策を準備しておく必要があるのだが，多くの日本人にはまだそのような習慣はない。
・今後，わが国における海外渡航者のためのワクチンの環境整備が向上するとともに，海外渡航者が事前に渡航地の感染症情報を収集し，必要な感染症対策を準備する習慣をもてるよう啓発していくべきである。

はじめに

近年，わが国の海外渡航者数は増え続け，2012年には年間1800万人以上となった。一方，海外からの訪日外国人旅行者数も増加し，2013年に初めて年間1000万人を超えている。渡航先や旅行形態にも変化がみられ，仕事のため家族連れで長期間途上国に赴任する場合や，既存の観光地のみならず冒険旅行などのように従来とは異なる地域に足を踏み入れる場合なども多くなっており，海外渡航者がさまざまな感染症に罹患する危険性が増しており(**表1**)[1]，日本には存在しない感染症が国内に持ち込まれるリスクも高くなってきている。

欧米諸国では，海外渡航者の健康問題を扱う医療機関としてトラベルクリニックが数多く設置されており，健康指導，ワクチン接種や携帯医薬品の処方などが行われているが[2]，わが国においては都市部ではトラベルクリニックは増えているものの地方ではまだ少なく，地域によっては海外渡航時のワクチンを接種できる医療機関がほとんどないという場合も珍しくないのが現状である。本来，海外渡航をする人は渡航地の感染症情報や治安状況を事前に調べ，ワクチン接種をはじめとする必要な感染症予防対策を準備しておく必要があるのだが，残念ながら多くの日本人にはまだそのような習慣はない。

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