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Molecular Epidemiological Study of *Moraxella catarrhalis* Isolated from Nosocomial Respiratory Infection Patients in a Community Hospital in Japan

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Abstract

Background *Moraxella catarrhalis*, occasionally, plays the essential role in nosocomial respiratory infection (NRI). Few studies have reported the route by which this organism spreads in a nosocomial infection outbreak. We identified characteristics of the strains isolated from NRI and attempted to reveal the potential nosocomial transmission routes.

Methods A follow-up study has been performed in a Japanese community hospital between July 2002 and January 2003. *M. catarrhalis* clinical isolates were identified and β-lactamase production test as well as the minimal inhibitory concentrations (MICs) have been examined. Pulsed-field gel electrophoresis (PFGE) and the multi locus sequence typing method (MLST) have been introduced as the effective "fingerprinting" methods.

Results A total of 29 strains were isolated from 17 participants; 7 independent DNA fragment patterns were detected by PFGE. Pattern B (defined in this study) was dominant, and was detected both in strains from a health care worker (HCW) and inpatients. In the 9 selected strains analyzed by MLST, 7 unique MLST types were identified, which showed the congruence with the results of PFGE results.

Conclusion Epidemiological analysis proved the transmission route from patient to patient, and suggested that more studies should be focused on identifying the possible transmission route between HCWs and inpatients.

Key words: *Moraxella catarrhalis*, nosocomial respiratory infection, nosocomial transmission route, PFGE, MLST

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Introduction

Moraxella catarrhalis causes human respiratory infection, which has been reported as one of the main pathogens of community-acquired pneumonia (CAP) (5, 11, 34, 1, 21, 32). *M. catarrhalis* also plays an important role in nosocomial respiratory infection (NRI) (4, 14, 19, 20), and spread between inpatients and outpatients, which have been previously demonstrated. Many aged inpatients are thought to have a compromised immune system. Whether or not they occasionally acquire *M. catarrhalis* colonization from the health care workers (HCWs) and subsequently developed a NRI is still controversial (19). The means by which this pathogen becomes an epidemic in hospitals, remains unclarified; to date the available evidence is limited. It remains a high priority to reveal the nosocomial transmission route.

Pulsed field gel electrophoresis (PFGE) is regarded as one of the credible "fingerprinting" methods, which has been widely used to investigate the spreading of *Streptococcus pneumoniae*, *M. catarrhalis* and many other pathogens (17, 19, 25, 26, 31, 33). Multi locus sequence typing (MLST) is

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a newly developed method, which is a nucleotide sequence-based approach for the unambiguous characteristics of isolates via the Internet (2, 18, 29), thus it has a great sensitivity due to its ability to detect neutral genetic variations (8, 22). MLST has been successfully widely used for analysis of many common pathogens such as *Neisseria meningitidis*, *S. pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Campylobacter jejuni* (6, 7, 9, 10), but not for *M. catarrhalis*.

In this study, we investigated the characteristics of *M. catarrhalis* clinical isolates and detected the possible nosocomial transmission route by PFGE and MLST.

Materials and Methods

Research site

Tagami Hospital is a community hospital affiliated with Nagasaki University, which is located in Nagasaki, Japan. There are 180 beds, 5 floors, and 9 wards, including the outpatient department (first floor), 41 surgery beds (second floor), 53 internal medicine beds (third floor), and long-term-care wards with 86 beds (fourth floor, 43 beds; fifth floor, 43 beds). Additionally, male and female patients are in separate wards.

Participants

From July 2002 to January 2003 (7 months), 14 patients and 3 HCWs were recruited; 6 were females, 11 were males (ratio, 0.55: 1), and the mean age was 70.4 years (range, 22 to 99). During their admission 12 inpatients had pneumonia; they were diagnosed as NRI, the main underlying diseases were chronic obstructive pulmonary diseases, and aspiration bronchitis. Three outpatients were diagnosed as CAP. [Patient (P-4) was counted as an outpatient and then as an inpatient after his admission.]

NRI was defined according to the CDC recommendation (12). Pneumonia was diagnosed if there was an appearance of a new abnormal shadow and likely infiltration on a chest roentgenogram and if at least two of the following clinical and laboratory findings were presented: fever (temperature> 37.8℃), cough, production of purulent sputum, dyspnoea, and leukocytosis (WBC count>10,000/mL).

Bacterial strains

Gram-stained smears and cultures of good quality specimens, obtained as recently as possible, were performed to identify *M. catarrhalis* isolates. Regarding the strains, 26 were from sputum, 2 were isolated from the pharynx, and 1 was from the nasal cavity. 19 strains isolated from inpatients were defined as primary causative pathogens of NRI. Isolates were inoculated on 5% blood agar plates and cultured at 37℃ in 5% CO_2 for overnight. β-lactamase production was detected by means of a disc impregnated with nitrocefin (Becton Dickinson, Sparks, MD, USA).

Antimicrobial susceptibility test

Minimal inhibitory concentrations (MICs) were determined by the agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute (23). All isolates were tested for susceptibility to the following 4 antibiotics: penicillin G (PCG) (Meiji Seika Kaisha, Tokyo, Japan), amoxicillin/clavulanate potassium (AMPC/CVA) (GlaxoSmithKline Co., Middlesex, UK), cefditoren (CDTR) (Meiji Seika Kaisha, Tokyo, Japan), erythromycin (EM) (Dainippon Pharmaceutical Co., Osaka, Japan).

PFGE

One colony was inoculated into 4 mL of brain heart infusion (BHI) broth, and cultured at 37℃ for overnight. PFGE with Spe I (Takara Bio Inc., Shiga, Japan) chromosomal digestion was performed to determine genetic relatedness, as described previously (16, 33), and the interpretation of PFGE patterns was based on the criteria described by Tenover et al. (28).

Genomic DNA preparation and sequence analysis

The internal genomic fragments of 8 house-keeping genes, *ppa* (pyrophosphate phospho-hydrolase), *efp* (elongation factor P), *fumC* (fumarate hydratase), *trpE* (anthranilate synthase component I), *mutY* (adenine glycosylase), *adk* (adenylate kinase), *abcZ* (ATP-binding protein), *glyRS* (glycyl-tRNA synthetase beta subunit) were recommended for MLST. Primers are available from the MLST web site (http://web.mpiib-berlin.mpg.de/mlst/dbs/Mcatarrhalis/documents/primers Catarrhalis_html).

PCR was performed in volumes of 50 μL, with an initial denaturation at 95℃ for 1 minute, followed by 30 cycles of 95℃ for 20 sec, 52℃ for 20 sec (except for *glyRS* 58℃ and *adk* 54℃), and 72℃ for 20 sec, and a final extension of 72℃ for 10 minute (TakaRa Ex Taq Hot Start Version, TaKaRa Bio inc., Shiga, Japan). PCR productions were cleaned up using Wizard SV Gel and PCR Clean-Up System (Promega Co., Madison, WI, USA). All Amplified DNA fragments were achieved using an Applied Biosystems Prism 377 automated DNA sequencer with BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems Co., Foster City, CA, USA). Sequences were assembled using OMIGA 2.0 software (Oxford Molecular Group Inc., Oxford, UK).

MLST

A differed sequence was assigned as a distinct allele, and the allelic profiles of the 8 loci defined as MLST sequence type (ST). STs gained after nucleotides sequences uploaded and analyzed via MLST database (http://web.mpiib-berlin.mpg.de/mlst/dbs/Mcatarrhalis/). The relatedness among the strains was identified by constructing a dendrogram based on the pair-wise differences in the allelic sequences by the unweighted pair group method (UPGMA) with arithmetic averages. The distance matrix was generated from the set of allelic profiles using Phylogenetic tree graph in this study.

Table 1. Characteristics of 29 *M. catarrhalis* Strains

No.	Stock No.	Sampling Date	Groups	Participants	Age	Gender	Sample	β-lactamase	MICs (μg/mL)*				PFGE	Diagnosis**
									PCG	CVA/AMPC	CDTR	EM		
1	KT-1	02.7.11	HCWs	P-1	38	F	Pharynx	+	0.5	0.032	0.032	0.25	A	-
2	KT-2	02.11.13	HCWs	P-2	22	M	Sputum	+	16	0.125	0.25	0.25	B	-
3	KT-3	02.12.2	HCWs	P-3	39	M	Sputum	+	8	0.125	0.125	0.25	C	-
4	KT-4	02.12.2	HCWs	P-3	39	M	Nasal cavity	+	8	0.125	0.125	0.25	C	-
5	KT-5	02.12.2	HCWs	P-3	39	M	Pharynx	+	8	0.125	0.125	0.5	C	-
6	KT-6	02.12.13	HCWs	P-2	22	M	Sputum	+	32	0.125	0.25	0.25	B	-
7	KT-7	02.12.19	HCWs	P-2	22	M	Sputum	+	16	0.125	0.125	0.125	B	-
8	KT-8	02.7.18	in (3F)	P-4	66	M	Sputum	+	16	0.25	0.5	0.063	D1	NRI
9	KT-9	02.7.29	in (2F)	P-5	69	F	Sputum	+	16	0.25	1	0.25	E	NRI
10	KT-10	02.8.2	in (3F)	P-6	96	M	Sputum	+	8	0.125	0.5	0.25	F	NRI
11	KT-11	02.10.24	in (5F)	P-7	98	M	Sputum	+	16	0.125	0.25	0.25	B	NRI
12	KT-12	02.10.28	in (5F)	P-8	72	F	Sputum	+	16	0.063	0.5	0.5	D2	NRI
13	KT-13	02.10.28	in (5F)	P-9	76	M	Sputum	+	32	0.125	0.25	0.25	B	NRI
14	KT-14	02.11.6	in (5F)	P-7	98	M	Sputum	+	16	0.125	0.125	0.25	B	NRI
15	KT-15	02.11.8	in (3F)	P-10	72	M	Sputum	+	8	0.063	0.25	0.25	B	NRI
16	KT-16	02.11.19	in (5F)	P-8	72	F	Sputum	+	32	0.125	0.125	0.25	B	NRI
17	KT-17	02.11.19	in (3F)	P-10	72	M	Sputum	+	8	0.063	0.5	0.25	B	NRI
18	KT-18	02.11.19	in (3F)	P-11	83	F	Sputum	+	8	0.063	0.25	0.25	B	NRI
19	KT-19	02.11.21	in (3F)	P-12	75	M	Sputum	+	16	0.063	0.25	0.25	B	NRI
20	KT-20	02.11.21	in (3F)	P-13	64	M	Sputum	+	16	0.063	0.25	0.25	B	NRI
21	KT-21	02.11.21	in (3F)	P-6	96	M	Sputum	+	2	0.032	0.063	0.063	E	NRI
22	KT-22	02.11.29	in (3F)	P-14	78	M	Sputum	+	8	0.063	0.5	0.25	B	NRI
23	KT-23	02.12.6	in (5F)	P-15	83	F	Sputum	+	8	0.063	0.5	0.5	D2	NRI
24	KT-24	03.1.7	in (5F)	P-7	99	M	Sputum	+	16	0.125	0.125	0.25	B	NRI
25	KT-25	03.1.10	in (3F)	P-6	97	M	Sputum	+	8	0.063	0.032	0.125	B	NRI
26	KT-26	03.1.29	in (5F)	P-7	98	M	Sputum	+	16	0.125	0.125	0.25	B	NRI
27	KT-27	02.6.21	out	P-4	66	M	Sputum	+	16	0.25	0.5	0.063	D1	CAP
28	KT-28	02.12.11	out	P-16	88	F	Sputum	+	4	0.032	0.125	0.25	G	CAP
29	KT-29	03.1.11	out	P-17	78	M	Sputum	+	16	0.063	0.25	0.5	D2	CAP

*PCG=penicillin G, AMPC/CVA=amoxicillin/clavulanate potassium, CDTR=cefditoren, EM=erythromycin.
**NRI=Nosocomial respiratory infection; CAP=Community acquired pneumonia.

(http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=treedraw&referer=pubmlst.org).

Results

Characteristics of M. catarrhalis isolates

From healthy HCWs, 7 strains were isolated; from inpatients and outpatients, 19 and 3 strains were recovered, respectively. All of the strains appeared to have the ability to produce β-lactamase. The respective range of MICs (μg/ML) against *M. catarrhalis* was 0.5-32 for PCG, 0.032-0.25 for AMPC/CVA, 0.032-1 for CDTR, and 0.063-0.5 for EM (Table 1). MICs seemed to have no influence on the severity of the illnesses, and NRI patients were cured or improved.

PFGE fingerprinting

Of the 7 distinguishable PFGE patterns which have been detected, pattern B (16, 55.2%) was dominant. Patterns A, B, and C were found in HCWs; B, D, E, and F were detected in inpatients; D and G were identified in outpatients. Patterns A, F and G were only detected from a single strain. In the HCWs group, 3 strains showed pattern B (from one HCW), 3 stains showed pattern C, and only strain KT-1 showed Pattern A. In the inpatients group, 13 strains (68.4%) showed pattern B, 3 strains showed pattern D, 2 strains showed pattern E, and only strain KT-10 showed pattern F. In the outpatient group, 2 of 3 strains showed pattern D, and the remaining strain showed pattern G (Fig. 1).

MLST typing analysis

Only one representative strain from each cluster that presented unique PFGE patterns was included in MLST analysis, except for 3 pattern B strains, because they were isolated from different groups or floors (Table 2). Allelic profiles identified 7 STs, all of which were newly detected and have been submitted to the online MLST database (http://web.mpiib-berlin.mpg.de/mlst/dbs/Mcatarrhalis/GetTableInfo_html).

Differing allelic profiles were found on at least 4 loci when alignment for each two STs was made; only one allelic profile of gene *trpE* showed uniformity (Table 2). Phylogenetic tree based on the matrix of pair-wise differences in the allelic sequences showed the linkage distance of all the STs, which indicated that no clonal relatedness existed among these strains (Fig. 2). Furthermore, KT-1 (ST 176, pattern A) and KT-9 (ST 178, pattern E) were aligned in the same cluster, which suggested that even though these strains belonged to different clones, they shared more common allelic sequences than others did.

KT-2, KT-11 and KT-15 presented identical ST, which confirmed the PFGE results and strongly suggested that these strains were the original from the same parent strain (Table 2).

Epidemiological analysis

Strains with PFGE pattern B were found both in HCWs and the inpatient group, which were described in Fig. 3. The first case, reported in the third week of October the P-7 patient might be the index case of pattern B strain dissemination. Until Nov. 6, pattern B strains were isolated only from the fifth floor, which suggested that the epidemic was limited to the male patient ward on the same floor. When the pattern B clone was detected from a radiographer (P-2) on

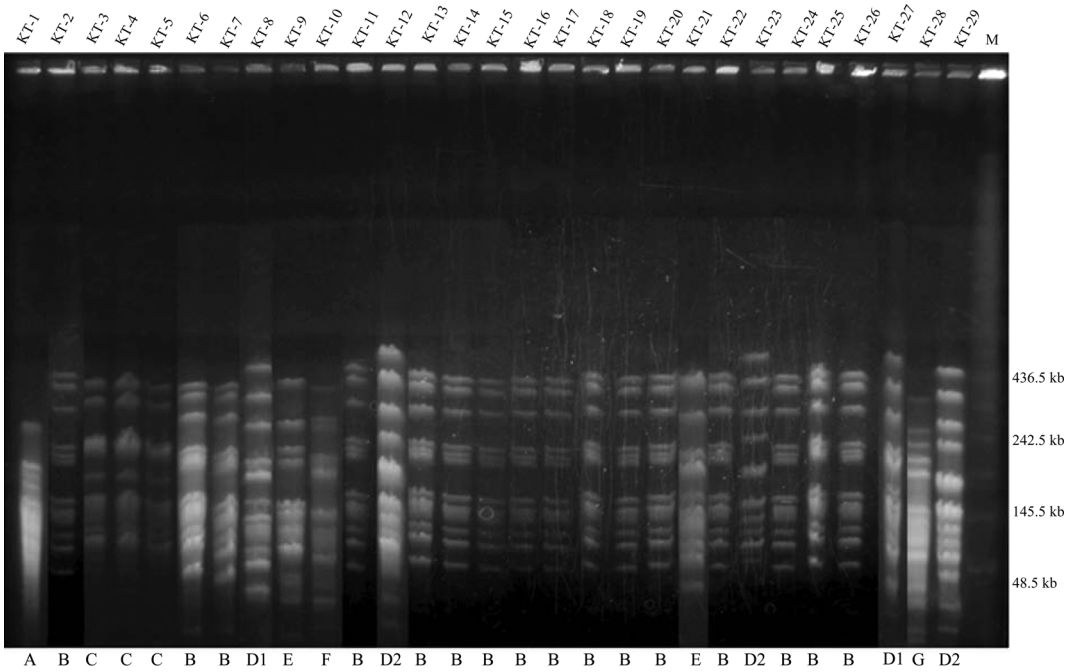

Figure 1. **PFGE profiles of all strains. DNA fragments were digested by *Spe I* restriction endonuclease, lane M contains a molecular size marker.**

Table 2. **MLST Allelic Profiles of Selected Strains**

Strain no.	Participants*	Sample source	PFGE Pattern	STs**	Genes							
					abcZ	*adk*	*efp*	*fumC*	*glyRS*	*mutY*	*ppa*	*trpE*
KT-1	S	Pharynx	A	ST 176	9	8	3	7	14	3	3	2
KT-8	In (3F)	Sputum	D1	ST 177	22	3	2	18	29	31	3	2
KT-9	In (2F)	Sputum	E	ST 178	9	3	6	7	27	6	3	2
KT-10	In (3F)	Sputum	F	ST 179	3	22	6	2	17	9	25	2
KT-11	In (5F)	Sputum	B	ST 180	2	6	3	2	20	3	3	2
KT-15	In (3F)	Sputum	B	ST 180	2	6	3	2	20	3	3	2
KT-2	S	Sputum	B	ST 180	2	6	3	2	20	3	3	2
KT-3	S	Sputum	C	ST 181	3	3	6	7	15	15	9	2
KT-28	Out	Sputum	G	ST 182	8	6	12	2	50	26	2	2

*: S= Health care workers; In (2F)= inpatients living in 2nd floor, In (3F)= inpatients living in 3rd floor, In (5F)= inpatients living in 5th floor; Out=Outpatients. **: Multilocus sequence type.

Nov. 13, the epidemic was reported from not only the third floor but also in the female patient ward. One month later, and then a week after that, the radiographer provided sputum twice, and pattern B strains were detected in both samples.

KT-27, a pattern D strain was first detected from P-4 as an outpatient. From the same patient, KT-8 was isolated when he developed NRI after his admission, which showed the same PFGE pattern. During the following month, pattern D strains were also detected in the firth floor.

Discussion

Although many studies have revealed the nosocomial transmission route between hospital acquired infection patients and community acquired infection patients, there was still no direct evidence to verify whether HCWs as a risk factor plays the primary role in nosocomial infections, or to prove the prevalence of *M. catarrhalis* colonized in HCWs (19, 25, 27). Some studies reported that infants and children were colonized by *M. catarrhalis* at a higher rate than only 1-5% healthy adults did (21), but isolates from older patients were more likely to be pathogenically significant (32). In this study, no other HCWs showed *M. catarrhalis* after the clinical screening except for the radiographer (P-2) who was frequently taking the portable X-ray examination equipment in different wards during that period. He was possibly infected with pattern B strain due to very close contact with the NRI patients when he did the examinations. Since without presenting any clinical syndrome, he was considered to keep the colonization, which showed the same genetic pattern as isolates from patients. Unfortunately, we noticed that the evidence we present here is still insufficient to prove *M. catarrhalis* spreading between inpatients and HCWs; more epidemiological information about *M. catarrhalis* isolates from HCWs and health residences would be useful to discovery the truth. We suggest that more surveys should be focused on HCWs' behavior. Since direct-contact transmis-

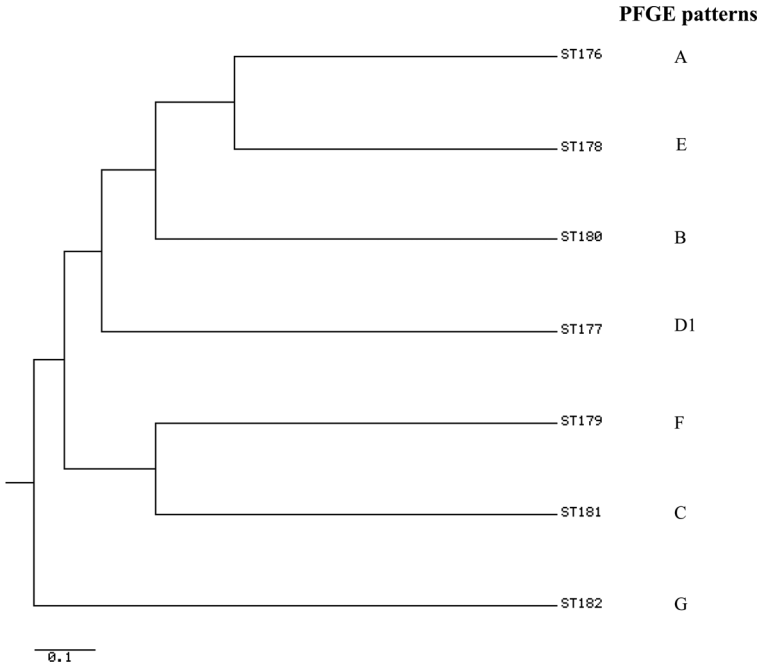

Figure 2. **Phylogenetic tree constructed from all the MLST allelic profiles showed genetic relationship and compared to the PFGE results. Linkage distance indicated by the scale at the bottom.**

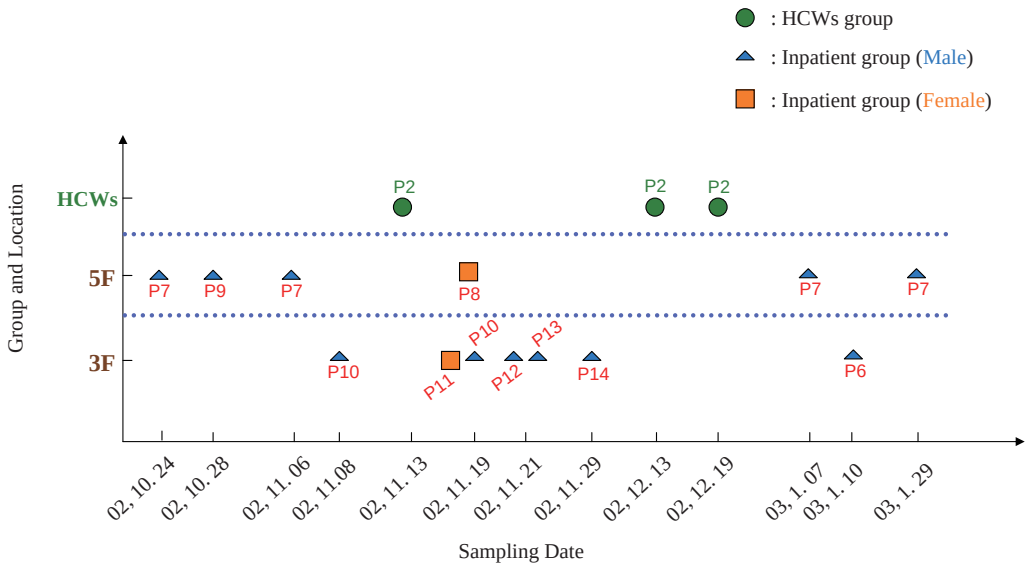

Figure 3. **Epidemiological characteristics of strains with PFGE pattern B.**

sion, droplet transmission, air-born transmission seemed to be the major transmission route in NRI (3), hand washing, glove using, mask/eye protection, face shield using, etc. should be advocated as the standard precaution when HCWs perform medical practice.

Sma I and *Not I* were regarded as the suitable restriction endonuclease for PFGE (15, 19). We found less comparability of genetic characteristics digested by *Sma I*, because of few DNA fragment bands (data not shown). Genetic analysis based on *Not I* seemed to be better, nevertheless, for unknown reasons in several strains it was difficult to obtain PFGE bands as previous studies have discussed (19, 33). *Spe I* was regarded to produce readily comparable banding patterns (30), and thus was used in the present study.

The results showed the congruence between PFGE and MLST, which indicated that these methods were suitable techniques for verifying the clonal relatedness. MLST seemed to be capable to present more details compared to PFGE (Fig. 2). PFGE uniquely analyzes the whole chromosomal DNA and is comparatively cost-effective (13, 24). The most advantage of MLST is that allelic profiles are unambiguous and STs could easily be compared to those in many central databases via the Internet (10, 18, 22).

In conclusion, results of PFGE and MLST showed the congruence and were recommended to be useful in establishing genetic relatedness. We found evidence, which proved *M. catarrhalis* spreading from patient to patient, and suggested that more studies should be focused on the possi-

ble transmission route between HCWs and inpatients. Since HCWs might be a potential constructive factor in nosocomial infection, advocating standard precaution is very necessary for hospital acquired infection control and prevention.

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