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DNA restriction patterns produced by pulsed-field gel electrophoresis in *Moraxella catarrhalis* isolated from different geographical areas

G. MARTINEZ*, K. AHMED, C. H. ZHENG, K. WATANABE, K. OISHI AND T. NAGATAKE

Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

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SUMMARY

Pulsed field gel electrophoresis (PFGE) of the genomic DNA of *Moraxella catarrhalis* was done in 172 strains isolated from sputum of patients with respiratory infections in Nagasaki (130 strains), Europe (14 strains), Thailand (6 strains), Uganda (3 strains), Bangladesh (5 strains) and Kuwait (14 strains). Restriction endonuclease with *Sma*I generated 4–16 DNA fragments ranging from 1000 kb to 24·25 kb and was classified into 31 major groups. It was found that there were wide variations of DNA restriction patterns of strains isolated from the same and different geographical areas. DNA restriction patterns of strains isolated in Nagasaki during the last 12 years showed dynamic changes of the predominant strains in each time period. We conclude from this study that PFGE is a suitable method to document interstrain variation in *M. catarrhalis*.

INTRODUCTION

During the past decade *Moraxella catarrhalis* has become an established pathogen in humans, causing mainly respiratory and middle ear infections, in children as well as in adults. The world-wide distribution of this pathogen [1–5] as well as nosocomial outbreaks by *M. catarrhalis*, provide an opportunity for considering epidemiological studies of this bacterium. Several epidemiological typing methods have been used to characterize strains of *M. catarrhalis* such as phenotypic characterization, electrophoretic mobility of esterases, outer membrane proteins (OMPs), whole-cell proteins, plasmid profile, isoelectric focusing of β-lactamase enzymes, bacteriocin typing, haemagglutination, fimbriation and serotyping; however, these methods could not satisfactorily discriminate interstrain variability [5–12]. The restriction fragments length polymorphism (RFLP) has been used also for *M. catarrhalis*; however, the result was not satisfactory due to many small fragments which are difficult to compare.

Pulsed field gel electrophoresis (PFGE) is a powerful tool for species and strain identification, epidemiologic and ecologic studies because of the high sensitivity over other methods [9, 13, 14]. PFGE can resolve extremely large DNA, raising the upper size limit of DNA separation from 30 to 50 kb as in RFLP to well over 10 Mb. The large fragments of DNA generated by PFGE are easier to compare than many smaller fragments of DNA produced in RFLP [9]. Among several restriction endonucleases *Not*I and *Sma*I, were found to be suitable for PFGE of *M. catarrhalis* [13]. However, DNA of some strains of *M. catarrhalis* are refractory to digestion by *Not*I, because of the modification or methylation of the nucleoside(s) of the recognition sites [13]. Therefore, in this study PFGE was done on *Sma*I digested DNA of *M. catarrhalis* isolated from sputum of patients with respiratory infection in Nagasaki, Europe, Thailand, Uganda, Bangladesh and Kuwait to determine the relatedness among strains.

* Author for correspondence: Department of Internal Medicine, Institute of Tropical Medicine 1-12-4 Sakamoto machi, Nagasaki 852-8102, Japan.

MATERIAL AND METHODS

Bacterial strains and growth condition

All strains used in this study were isolated from patient with respiratory tract infection. From 2573 strains of *M. catarrhalis* isolated in Nagasaki during the last 12 years (1984–96), 130 strains were randomly selected from the stock of our laboratory. Strains isolated in different periods of time, from Thailand (6 strains), Bangladesh (5 strains), Kuwait (14 strains), Uganda (3 strains) and Europe (14 strains) were also obtained from the collection of our laboratory. European isolates contained 11 strains from Belgium and 3 strains each from Netherlands, Sweden and Scotland. *M. catarrhalis* was identified based on the colony appearance, Gram-negative diplococcal morphology, DNase production detected by DNase Test Agar (DIFCO Laboratories, Detroit, MI, USA) and standard biochemical tests [15]. Bacteria were maintained in Brain Heart Infusion (BHI) broth (BBL Microbiology System, Bectom Dickinson and Co., Cockeysville, MD, USA) containing 5% horse blood at −40 °C until use.

Pulsed field gel electrophoresis

M. catarrhalis was cultured on BHI agar (BBL Microbiology System, Bectom Dickinson and Co., Cockeysville, MD, USA) for 18 h at 37 °C in a 5% CO_2 incubator, then three colonies were subcultured in 3 ml of BHI broth (BBL Microbiology System, Bectom Dickinson and Co., Cockeysville, MD, USA) and incubated at 37 °C overnight. *M. catarrhalis* were harvested by centrifuging 1 ml of BHI broth, at 4300 ***g*** in 4 °C for 5 min, then washed with saline-EDTA (0·15 M NaCl–10 mM EDTA, pH 8·0) by centrifugation.

To the pellet, 200 μl of Pett IV solution (1 M NaCl–10 mM EDTA, pH 8·0) was added and kept at 37 °C. From this suspension 120 μl was taken and added to an equal volume of melted agarose (2% clean cut agarose, Biorad Laboratories, CA, USA). To this 1 μl (5 mg/ml) of lysozyme (Taiyou Kagaku, Mie, Japan) was also added, the mixture was poured into a plug mould (Bio-Rad Laboratories, CA, USA), chilled at 4 °C for 20 min. The plug was removed and was treated with 1 ml of lysis solution (1 M NaCl–0·1 M EDTA, pH 8·0–10 mM Tris–HCl, pH 8–0·5% wt/vol Brij 58–0·2% wt/vol deoxycholate–0·5% wt/vol Sarkosyl) plus 1 μl (5 mg/ml) of lysozyme for 1 h at 37 °C. Subsequently the plugs were treated with 1·5 ml of ES solution (0·25 M EDTA pH 8·0–1% wt/vol Sarkosyl) plus 0·1 mg/ml Proteinase K (Wako Pure Chemical Industries Ltd, Osaka, Japan) and kept at 50 °C for 15 h. Then the solution was removed and 1·5 ml of TE buffer (10 mM Tris–HCl pH 8·0–1 mM EDTA pH 8·0) plus 1 mM phenylmethysulfonyl fluoride was added and kept at room temperature for 4 h. Plugs were washed three times with cold (4 °C) TE buffer each time for 10 min. Prior to digestion each plug was immersed in TE buffer for 20 min at room temperature. After removing TE buffer, 1·5 ml of *Sma*I buffer (10 mM Tris–HCl pH 8·0–7 mM MgCl–20 mM KCl–7 mM 2 mercaptoethanol) was added. Finally the *Sma*I buffer was removed and 1 μl (10 units) of *Sma*I enzyme (Takara Shuzo Co., Ltd, Shiga, Japan) in 80 μl of *Sma*I buffer was added and kept at 30 °C overnight. PFGE was done with Gene Navigator System Pharmacia Biotech, Sweden) at 170 V, 400 amp for 33 h at 8 °C. Pulse time was divided into 3 phases; 10 s for 11 h, 25 s for 11 h, 75 s for 11 h. Gel staining was done by ethidium bromide and photography was by a Polaroid camera. Sizes of DNA fragments were determined by measuring distance of band migration compared with the DNA standards of a Lambda DNA ladder (Bio-Rad Laboratories, CA, USA).

PFGE was done a total of 172 strains of *M. catarrhalis*. From all strains, 31 major patterns were obtained denominated by alphabetical letters. Strains which were possibly related (PR) or closely related (CR) to each group were sub-grouped and was denominated by the alphabetical letter of the group followed by a number. The banding patterns were compared according to the method of Tenover and colleagues, that refer strains as indistinguishable if the number of fragment differences compared with the reference pattern is 0, closely related if the fragment differences are 2–3, possibly related if the fragment differences are 4–6, and different if more than 7 fragments are different [16]. To compare the banding patterns, a strain isolated in 1984 from this laboratory was taken as a reference.

RESULTS

M. catarrhalis isolated from Nagasaki generated four patterns grouped as A (19 strains), B (14 strains), C (1 strain), E (8 strains) and one pattern of sub-group D. CR and PR patterns to group A were denominated as A1 (13 strains) and A2 (6 strains) respectively. Other PR subgroup were B1 (12 strains), B2 (14 strains), D1

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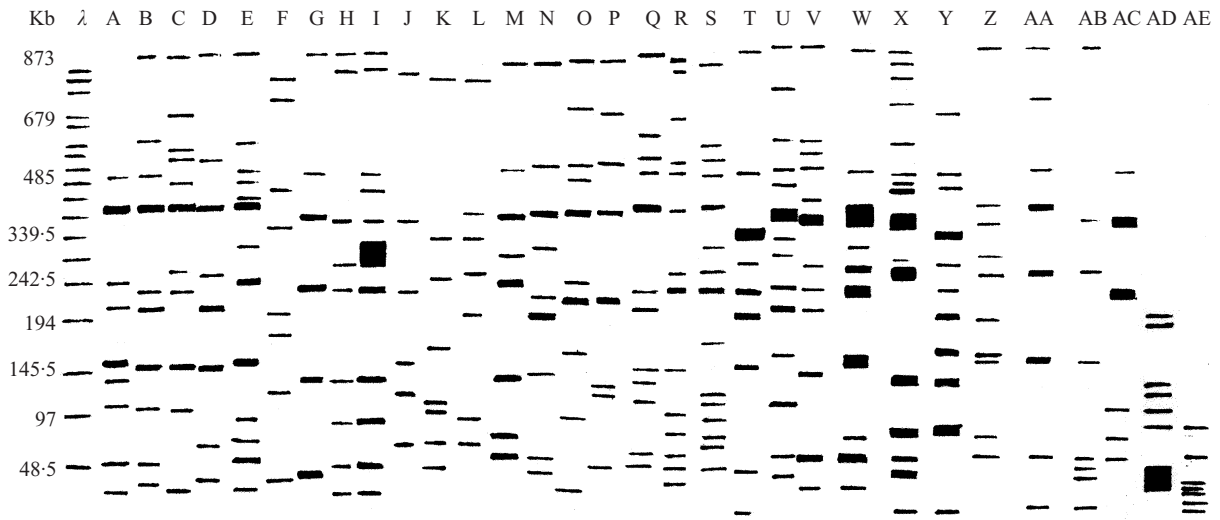

Fig. 1.

Table 1. *Group and sub-group patterns of* M. catarrhalis *generated by PFGE*

Area	Group	Sub-group*
Nagasaki	A (19 strains)	A1 (13 strains) CR, A2 (6 strains) PR
	B (14 strains)	B1 (12 strains) PR, B2 (14 strains) PR
	C (1 strain)	
		D1 (11 strains) PR, D2 (9 strains) PR
		D3 (8 strains) PR, D4 (10 strains) PR
		D5 (5) PR
	E (8 strains)	
Thailand	F (2 strains)	F1 (1 strain) PR, F2 (1 strain) PR
		F3 (1 strain) PR, F4 (1 strain) PR
Uganda	G (1 strain)	
	H (1 strain)	
	I (1 strain)	
Bangladesh	J (1 strain)	J1 (1 strain) PR, J2 (1 strain) PR
	K (1 strain)	
	L (1 strain)	
Europe	D (2 strains)	D1 (1 strain) PR, D2 (1 strain) PR
	M (1 strain)	M1 (1 strain) PR, M2 (1 strain) PR
		M3 (1 strain) PR
	N (1 strain)	
	O (1 strain)	
	P (1 strain)	
	Q (1 strain)	
	R (1 strain)	
	S (1 strain)	
Kuwait	T, U, V, W, X, Y, Z, AA, AB (1 strain of each one)	
	AC (1 strain)	
	AD (1 strain)	
	AE (1 strain)	

* CR, closely related; PR, possibly related.

(11 strains), D2 (9 strains), D3 (8 strains) D4 (10 strains) and D5 (5 strains).

Strains isolated from Thailand were denominated as F group (2 strains). There were 4 PR strains which were different from each other and were designated as F1 (1 strain), F2 (1 strain), F3 (1 strain) and F4 (1

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Table 2. *Common DNA fragments of* M. catarrhalis *isolated from different geographical areas*

Country strains	Kb fragment									
Nagasaki	873			388		145·5				
Thailand		824·5	485		339·5		121·25			
Uganda	873				339·5		121·25			
Bangladesh		824·5						72·75		
Europe	873			388						
Kuwait	873 (8/14)							48·5 (11/14)		

Table 3. *Distribution of different electrophoretic patterns of group and sub-group of PFGE generated by* Sma*I of* M. catarrhalis *during 1984–96 in Nagasaki*

	Group				Sub-group								
Year	A	B	C	E	A1	A2	B1	B2	D1	D2	D3	D4	D5
1984	8*							2					
1985	4												
1986	2	3		1	1								
1987	3	2			1	1	2	2					
1988			1		1	1	1	3					
1989	2	4		1	1		1		2	2			
1990		2		1	1		1	1	4	4			
1991		3		1	1		1	1	5	3			
1992				1	2		1	2			1	2	1
1993				1	1	1	2				2	2	1
1994				1	1	1	1				3	3	1
1995				1	1	1	1	2			1	1	1
1996					2	1	1	1			1	2	1

* Frequency of patterns.

strain). There was a total of 3 strains from Uganda, all were different from each other and denominated as G, H and I group. Five strains from Bangladesh were available and generated 3 patterns denominated as J, K and L. PR to J group were 2 strains, sub-grouped as J1, and J2. Fourteen European strains generated 6 major patterns, denominated as D, M, N, O, P, Q, R and S. PR to D were 2 strains designated as D1, D2. PR to group M (1 strain) there were 3 strains M1, M2 and M3. Group N, O, P, Q, R and S had 1 strain in each group. Kuwait strains (14 strains) were classified into 12 major patterns, T, U, V, W, X, Y, Z, AA, AB, AC, AD and AE. Only 2 strains sub-grouped as AC1 and AC2 were PR to the group AC (Table 1).

*Sma*I generated 4–16 fragments after digestion. The estimated molecular size of the largest and smallest fragments were 1000 kb and 24·25 kb, respectively. According to the DNA fragments in Table 2: Nagasaki strains were sharing 3 fragments of 873, 388 and 145·5 kb. Thailand strains shared 4 common fragments of 824·5, 485, 339·5 and 121·25 kb. Although Ugandan strains were completely different, they shared however 3 common fragments of 873, 339·5 and 121·25 kb. The last 2 bands were also shared by Thailand strains. Five Bangladesh strains shared 2 fragments, 824·5 and 72·75 kb, sharing the first fragment with Thailand strains. European strains shared 2 common fragments, 873 and 388 kb these fragments were also common in Nagasaki strains. Kuwait strains were variable; no common fragment was found in all the strains, except the 873 kb fragment in 8 out of 14 strains and the 48·5 kb fragment in 11 out of 14 strains.

Yearly distribution of different groups and sub-groups showed that in the early years, Nagasaki strains were dominated by group A and B, which

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disappeared from 1990 and 1992, respectively (Table 3).

Group E, A1, A2, B1, and B2 appeared in 1987 and some of them appeared and disappeared in the subsequent years. Group D1 and D2 appeared in 1989 and disappeared in 1992; from this year, group D3, D4 and D5 appeared and continued as predominant strains.

DISCUSSION

The usefulness of PFGE for genomic DNA analysis is well established. It has been used in many bacteria to find out the interstrain relatedness [9, 10]. However, for *M. catarrhalis*, studies using PFGE are few [13, 17]. One of the studies was done in 38 clinical isolates of this bacteria at Shinshu University Hospital, Nagano, Japan and the other study was done in order to construct a physical map of *M. catarrhalis*. The present study used the genomic DNA of *M. catarrhalis* to find out interstrain relatedness among the strains isolated from patients with respiratory infections in different geographical locations. Previously the OMPs of *M. catarrhalis* were used to find out interstrain variation of strains isolated from different geographical locations [12]. Interestingly, all strains generated homogenous banding patterns of the major OMPs. Our unpublished observations also showed similar results. The PFGE analysis in this study showed that there were much variations among the strains of *M. catarrhalis* isolated from same and different geographical locations. Therefore for interstrain variations minor bands of OMPs of *M. catarrhalis* may deserve consideration.

Though the number of strains analysed from areas other than Nagasaki were few, even they showed wide interstrain variations. Even the related strains in each sub-group were all possibly related except A1, which was the only closely related sub-group. In another study also, this wide variation of DNA banding patterns generated by PFGE was found among the clinical isolates of *M. catarrhalis* [13]. They presume from this variation that many of the isolates were derived from a common ancestor.

Although there were wide variations among the strains isolated from the same locality, there were some common bands shared by the strains. These bands may act as a marker for the origin of the strains. Further studies regarding these bands are necessary, which may explain some of the major gaps in our understanding of this organism. All strains isolated at Nagasaki have common restriction fragments, which may suggest that these isolates have an ancestor strain and that they have been proliferating with slow genomic rearrangements. All strains isolated from each geographical area showed common fragments, except strains from Kuwait. More than half of the population of Kuwait are from different parts of the world and many travel frequently; this massive movement of people might bring strains from those areas. We assume this may be the reason why not a single fragment was shared by all the strains from Kuwait.

Analysis of the yearly distribution of different groups isolated in Nagasaki showed that some strains dominated in the early years and were gradually replaced by other strains. This 12 year analysis indicates that this change of strains may be an ongoing process, older types of strains are replaced by newer strains. We conclude from this study that there are wide interstrain variations among *M. catarrhalis* strains isolated from different geographical areas and even in the same area and that PFGE appears to be suitable for epidemiological studies of this bacterium.

REFERENCES

1. Utsunomiya Y, Ahmed K, Rikitomi N, et al. Isolation of pathogenic bacteria from induced sputum of hospitalized children with pneumonia in Bangladesh. J Trop Paed 1998: **44**: 338–42.
2. Ahmed K, Wilson S, Wafa YJ, et al. Quantitative and qualitative culture of sputum obtained from patients with respiratory tract infection in Kuwait. 1999 (Submitted for publication).
3. Nagatake T, Takahashi A, Tao M, et al. Causative organisms of acute respiratory infections in northern Thailand. Jpn J Trop Med Hyg 1993; **21**: 111–5.
4. Bealieu D, Scriver S, Bergeron MG, et al. Epidemiological typing of *Moraxella catarrhalis* by using DNA probes. J Clin Microbiol 1993; **31**: 736–9.
5. Verghese A, Berk LS. Introduction: *Branhamella catarralis*; a microbiologic and clinical update. Am J Med 1990; **88**: 1S.
6. Doern GV. *Branhamella catarrhalis*; phenotypic characteristics. Am J Med 1990; **88**: 33S.
7. Wallace RJ, Nash DR, Steingrube VA. Antibiotic susceptibilities and drug resistance in *Moraxella* (*Branhamella*) *catarrhalis*. Am J Med 1990; **88**: 46S.
8. Ahmed K, Rikitomi N, Matsumoto K. Fimbriation, hemagglutination and adherence properties of fresh clinical isolates of *Branhamella catarrhalis*. Microbiol Immunol 1992; **36**: 1009–101.
9. Lai E, Birren B. Use of secondary pulsed field gel electrophoresis in separation of large DNA. Annal. Biochem 1995; **224**: 68–74.

10. Shiimizu A, Kloos WE. Pulse-field electrophoresis of *Staphyloccocus hyicus* and *Staphylococcus chromogenes* genomic DNA and its taxonomic, epidemiologic and ecologic applications in veterinary medicine. J Vet Med Sci 1997; **59**: 443–50.
11. Carter GL, Wilson L, Dunn-Coleman NS. Molecular characterization of chromosomes by pulsed-field electrophoresis. Prog Industrial Microbiol 1994; **29**: 667–83.
12. Murphy TF, Bartos LC. Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarralis*. Infect Immun 1989; **57**: 2938–41.
13. Kawakami Y, Ueno I, Katsuyama T, Furihata K, Matsumoto H. Restriction fragment length polymorphism (RFLP) of genomic DNA of *Moraxella* (*Branhamella*) *catarrhalis* isolates in a Hospital. Microbiol Immunol. 1994; **38**: 891–5.
14. Patterson JE, Patterson TF, Farrel P, Hierholzer W, Zervos M. Evaluation of restriction endonuclease analysis as an epidemiologic typing system for *Branhamella catarrhalis*. J Clin Microbiol 1989; **27**: 944–6.
15. Isenberg HD. Essential procedures for clinical microbiology. Washington, D.C.: ASM press 1998: 54–5.
16. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995; **33**: 2233–9.
17. Furihata K, Sato K, Matsumoto H. Construction of a combined *Not*I/*Sma*I physical and genetic map of *M.* (*Branhamella*) *catarrhalis* strains ATCC25238. Microbiol Immunol 1995; **39**: 745–51.

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