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Molecular typing of *Mannheimia* (*Pasteurella*) *haemolytica* serotype A1 isolates from cattle in Japan

K. KATSUDA[1]*, M. KOHMOTO[2], K. KAWASHIMA[1], H. TSUNEMITSU[1], T. TSUBOI[3] AND M. EGUCHI[3]

[1] *Environmental Hygiene Section, Shichinohe Research Unit, National Institute of Animal Health, 31 Uminai, Shichinohe, Kamikita, Aomori 039-2586, Japan*
[2] *Pasturing Disease Section, Shichinohe Research Unit, National Institute of Animal Health, 31 Uminai, Shichinohe, Kamikita, Aomori 039-2586, Japan*
[3] *Clinical Epidemiology Section, National Institute of Animal Health, Tsukuba, Ibaraki 305-0856, Japan*

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SUMMARY

Pulsed field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) methods were applied for molecular typing of 130 *Mannheimia* (*Pasteurella*) *haemolytica* serotype A1 isolates obtained from 13 prefectures in Japan. These isolates were divided into 15 *Apa*I PFGE profiles that formed six distinct clusters (clusters A–F). Fifty-three (40·7%) isolates were classified in cluster B, and 20·0, 13·8, 12·3, 6·9 and 6·1% of isolates were in clusters E, A, F, D and C, respectively. The isolates of cluster B were differentiated into seven subtypes (B1–B7) and subtype B5 contained 63% (34/53) of isolates. RAPD revealed four banding patterns (types I–IV), and among 130 isolates 60·7% (79/130) of isolates were RAPD type I. All of the RAPD type I isolates were grouped into clusters A–C by PFGE. There was no relationship between molecular typing and geographic origin of these isolates. These results indicate that isolates of *M. haemolytica* A1 strain with various molecular profiles have already spread in Japan and may have caused sporadic infections.

INTRODUCTION

Mannheimia (*Pasteurella*) *haemolytica* is an important aetiological agent of pneumonic pasteurellosis of cattle, and causes considerable economic loss to the cattle industries [1]. To identify the routes of transmission of disease, epidemiological investigations are important. Although many techniques such as serotyping, plasmid profiling and/or antimicrobial susceptibility have been developed [2–4], these procedures have limitations. On *M. haemolytica*, it is reported that plasmid typing offers less reproducibility and only few serotypes are associated with this disease [7, 8]. A total of 13 capsular serotypes (designated A1, A2, A5–A9, A11–A14, A16 and A17) are now defined for this species [5, 6], but almost all the isolates from cattle developing pneumonia are serotype A1. Therefore, these methods are not adequate for epidemiological investigations and more highly discriminatory methods of typing are necessary.

In the last decade, molecular typing methods such as ribotyping (RT), chromosomal DNA digestion with either restriction enzyme analysis (REA) or pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA fingerprinting (RAPD) have been used in human and veterinary epidemiological investigations. These molecular typing methods provide more detailed epidemiological information on bacteria [9–14]. Recently, Kodjo et al. reported that

* Author for correspondence: Environmental Hygiene Section, Shichinohe Research Unit, National Institute of Animal Health, 31 Uminai, Shichinohe, Kamikita, Aomori 039-2586, Japan.

Table 1. *Results of PFGE molecular typing of* M. haemolytica *A1*

Prefecture	Year	PFGE	No. of isolates	RAPD	Source
Hokkaido	1991	B5	1	I	Nasal swab
	1995	B5	1	I	Nasal swab
	1995	B6	1	I	Nasal swab
	1995	F	1	IV	Nasal swab
Aomori	1993	B5	2	I	Lung
	1994	B5	2	I	Lung
Miyagi	1991	B5	2	I	Nasal swab
	1992	A1	2	I	Nasal swab
Ibaraki	1997	B5	2	I	Nasal swab
	1998	B5	2	I	Nasal swab
	1998	D	2	II	Nasal swab
	1999	E	2	III	Nasal swab
Gunma	1990	B2	2	I	Lung
	1993	A1	2	I	Nasal swab
	1993	B5	1	I	Lung
	1993	F	1	IV	Lung
	1994	B5	1	I	Lung
	1996	B5	1	I	Nasal swab
	1996	B5	1	I	Lung
	1996	B5	1	I	Nasal swab
	1996	E	5	III	Lung
	1996	E	1	III	Liver
	1996	E	1	III	Nasal swab
	1997	B5	1	I	Lung
	1998	B1	1	I	Lung
	1999	B5	1	I	Blood
	1999	B3	1	I	Nasal swab
Chiba	1990	B5	1	I	Lung
	1999	A3	2	I	Nasal swab
	1999	B5	2	I	Nasal swab
	2000	D	3	II	Nasal swab
Tochigi	1997	B5	2	I	Nasal swab
	1998	B5	2	I	Nasal swab
	1998	E	2	III	Nasal swab
	1999	D	2	II	Nasal swab
Niigata	1998	B5	1	I	Nasal swab
Aichi	1993	B5	2	I	Nasal swab
	1994	A1	2	I	Nasal swab
Hyogo	1994	A3	1	I	Lung
	1994	F	1	IV	Lung
	1994	F	1	IV	Lung
	1994	F	1	IV	Lung
	1995	B5	1	I	Lung
Hiroshima	1996	B5	2	I	Lung
	1997	B4	2	I	Lung
Yamaguchi	1984	F	1	IV	Lung
	1987	B4	3	I	Nasal Swab
	1987	C1	1	I	Lung
	1988	A3	3	I	Nasal Swab
	1988	C2	3	I	Lung
	1989	C2	3	I	Nasal Swab
	1989	C2	1	I	Lung
	1991	B7	3	I	Nasal Swab
	1995	D	2	II	Lung
	1996	A2	2	I	Nasal Swab

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Table 1 (*cont.*)

Prefecture	Year	PFGE	No. of isolates	RAPD	Source
	1996	A2	2	I	Nasal Swab
	1996	A2	2	I	Nasal Swab
	1996	B5	1	I	Lung
	1997	B6	3	I	Lung
	1997	E	3	III	Nasal Swab
	1998	B1	3	I	Nasal Swab
	1998	E	3	III	Nasal Swab
	1998	E	6	III	Lung
	1998	E	3	III	Lung
Fukuoka	1997	F	1	IV	Lung
	1997	F	6	IV	Nasal Swab
	1997	F	2	IV	Lung
	1997	B5	2	I	Lung

PFGE is more useful than RT and RAPD for *M. haemolytica* epidemiological investigations [15]. However, they tested only nine isolates of serotype A1 from cattle and there are only a few reports regarding application of molecular typing methods to survey on this bacterium [10, 13]. In this study, we applied RAPD and PFGE methods to an extensive epidemiological study on *M. haemolytica* serotype A1 isolates in Japan.

MATERIAL AND METHODS

Bacterial isolates

The isolates investigated in this study are listed in Table 1. A total of 130 *Mannheimia haemolytica* A1 field isolates from 68 herds affected with pneumonic pasteurellosis within 13 prefectures were obtained between 1984 and 2000 in Japan. Serotyping was conducted by slide agglutination tests [16] with serotype A1 specific antiserum.

RAPD analysis

In preliminary experiments, we tested 41 oligonucleotides. Primer PB-1 (5′-ggAACTgCTA-3′) that yielded reproducible and clear profiles was selected to use in following procedure. The RAPD reaction was performed in 25 μl volume containing 3 mM $MgCl_2$; 10 mM Tris-HCl (pH 8·3); 50 mM KCl; 0·1% Triton X-100; 250 μl M each dNTP; 20 pmol primer; 30–80 ng DNA; and 1 U of *Taq* polymerase (Takara Co. Ltd). Amplification was carried out in a DNA Thermalcycler (GeneAmp PCR System 2400 Perkin-Elmer) programmed for 5 cycles of 5 min at 94 °C, 5 min at 45 °C and 5 min at 72 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C. For profile analysis, 10 μl of amplification products were loaded on 2% agarose gels in TBE buffer and run at 100 V for 3 h, visualized by ethidium bromide staining and photography under UV light.

PFGE analysis

PFGE was performed according to Matushek et al. [17] with minor modification. Briefly, for chromosomal DNA extraction, an overnight agar culture was inoculated into 3 ml of brain heart infusion broth (Difco Laboratories, Inc., USA) and incubated with shaking at 37 °C for 2·5 h. Afterwards, cells were harvested and washed once in 1 ml of TEN solution (0·1 M Tris, 0·15 M NaCl, 10 mM EDTA pH 7·5). The cells were resuspended in 110 μl of EC lysis solution (1 M NaCl, 0·1 M EDTA, 6 mM Tris-HCl, 0·5% Briji 58, 0·2% deoxycholate, 0·5% sarkosyl and 0·3 mg/ml lysozyme, pH 7·5). The suspension was mixed with an equal volume of 1·0% low-melting-temperature agarose prepared with EC lysis solution and was allowed to solidify in block. The block was incubated at 37 °C for 1 h in 1 ml of EC lysis solution. The block was incubated at 50 °C for 1 h in 1 ml of ES solution supplemented with proteinase K (0·5 mg/ml) and was then treated twice in 1 ml of 1 mM PMSF in TE buffer at room temperature for 30 min and washed four times with TE buffer. Thinly sliced sections of block were digested with 25 U of *Apa*I (New England Biolabs, Inc.) for 1 h. The digested DNA was electrophoresed

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through a 1·0% PFGE agarose gel (Bio-Rad Laboratories, Ontario, Canada) in TBE buffer [0·1 M Tris, 0·1 M boric acid, 2 mM EDTA (pH 8·0)] by using the CHEF DR-II system (Bio-Rad Laboratories). The conditions for electrophoresis were: 6 V/cm at 14 °C, with pulse times ranging from 1–10 s for 9 h and then with pulse times ranging from 10–80 s for 11 h. Lambda ladders (New England Biolabs, Inc.) was used as size markers. DNA was visualized by ethidium bromide staining and photography under UV light.

Data analysis

The similarities among PFGE profiles were calculated by the Dice similarity index [18, 19] and Diversity Database Software (PDI, Huntington Station, NY, USA). The Dice coefficients (S_J) of band-based similarity were calculated as $S_J = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of bands common for A and B, and N_A and N_B are the total number of bands in A and B, respectively. The Dice coefficient was chosen to give more weight to matching bands. Dendrograms based on results of the matrix of similarity values were created with the unweighted-pair group method by using average linkages clustering.

RESULTS

RAPD analysis

All isolates that reacted with antiserum against type A1 were analyzed by RAPD with primer PB-1. Among the 130 isolates, RAPD gave four independent patterns, designated types I–IV. The primer yielded 10–16 amplified products ranging in size from 0·26–1·85 kb. Each profile type is shown in Figure 1. The isolates from each herd reveal an identical RAPD profile.

Seventy-nine isolates from 46 herds were classified into type I, nine isolates from four herds were into type II, 26 isolates from nine herds were into type III and 16 isolates from nine herds were into type IV (Table 1). The reproducibility of the RAPD method was examined in all isolates by four independent reactions. No inconsistency was observed in any of the four independent experiments (data not shown).

PFGE analysis

Genomes of *M. haemolytica* were digested with restriction endonuclease *Apa*I, *Sma*I, *Not*I and *Xba*I,

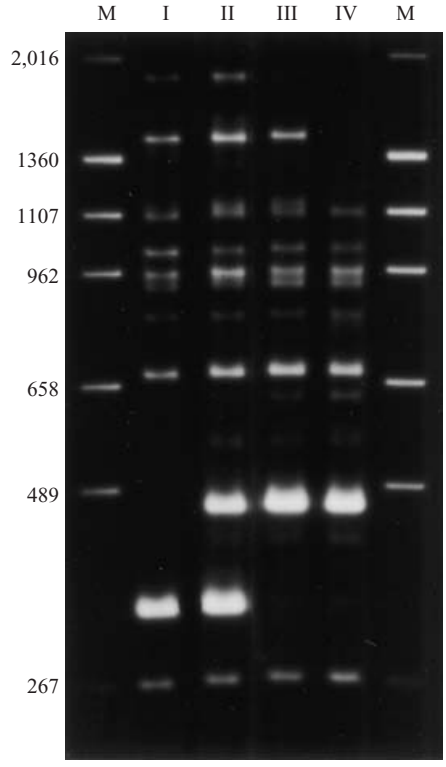

Fig. 1. RAPD pattern of *M. haemolytica* serotype A1. Lanes 2 through 5, RAPD types I through IV, respectively; lane 1 and 10, molecular size standard (pHY marker).

respectively (data not shown). *Apa*I yielded 14–18 fragments ranging from 15–1000 kb (Fig. 2*a*), which was suitable for PFGE analysis to compare *M. haemolytica* isolates from each other. One hundred and thirty isolates were analysed by PFGE with *Apa*I digestion, and all of the involved isolates revealed PFGE profiles. The isolates from each herd represented an identical PFGE profile (Table 1). No differences were recognized among the genotypes of *M. haemolytica* A1 isolated from nasopharynx and lung lesions of identical cattle of eight herds (data not shown).

The PFGE generated 15 independent profiles that formed six distinct clusters arbitrarily identified as A–F (Fig. 2*b*). Fifty-three isolates (40·7%) from 33 herds belonged to cluster B and 26 (22·0%) from nine herds, 18 (13·8%) from nine herds, 16 (12·3%) from nine herds, 9 (6·9%) from four herds and 8 (6·1%) isolates from four herds were identified as clusters E, A, F, D, and C respectively. The isolates belonging to cluster A were discriminated into three subtypes (A1–A3), cluster B was into seven subtypes (B1–B7), and cluster C was into two subtypes (C1 and C2) (Fig. 2*b*). Subtype B5 was the most prevalent (34 isolates from 24 herds) and found in each district

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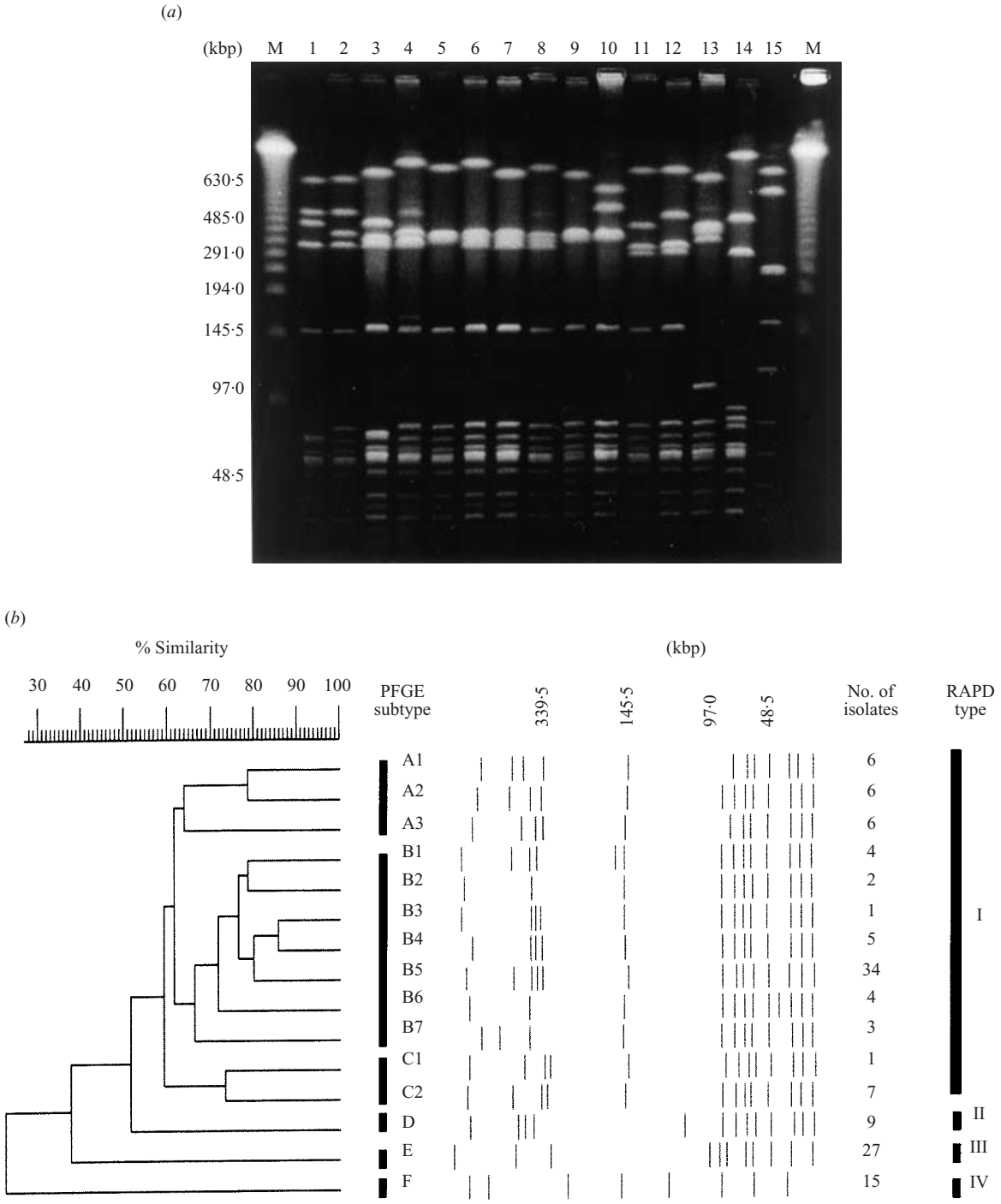

Fig. 2. (*a*) PFGE pattern of *M. haemolytica* serotype A1 with *Apa*I digestion. Lanes: M, lambda 48·5-kbp ladder; 1, A1; 2, A2; 3, A3; 4, B1; 5, B2; 6, B3; 7, B4; 8, B5; 9, B6; 10, B7; 11, C1; 12, C2; 13, D; 14, E; 15, F. (*b*) Dendrogram and schematic representation of PFGE fragments of *M. haemolytica* serotype A1 with *Apa*I digestion. Similarity analysis was performed using the Dice coefficient, and clustering was by UPGMA.

(13 prefectures) investigated in this study. The percent of similarity of clusters B–F to cluster A according to the Dice coefficient were 62, 59, 52, 38 and 23% respectively.

We associated the PFGE cluster with the year of isolation and the geographical origins (Table 1). Except for eight isolates that belonged to cluster C, there was no relation among PFGE cluster and geographical origins of isolates. All isolates in cluster C originated in Yamaguchi prefecture during 1987–1989. Before 1994, all isolates except one were grouped into clusters A, B or C, and the number of

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Table 2. *Prevalence of PFGE types over time*

	No. (%) of isolates						
Year	A	B	C	D	E	F	Total
1984–1990	3 (16·7)	6 (33·3)	8 (44·4)	0 (0·0)	0 (0·0)	1 (5·5)	18
1991–1993	4 (26·7)	11 (73·3)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	15
1994–1996	9 (25·7)	12 (34·3)	0 (0·0)	2 (5·7)	7 (20·0)	5 (14·3)	35
1997–1998	0 (0·0)	21 (42·8)	0 (0·0)	2 (4·1)	17 (34·7)	9 (18·4)	49
1999–2000	2 (15·4)	4 (30·8)	0 (0·0)	5 (38·5)	2 (15·4)	0 (0·0)	13
Total	18	53	8	9	26	16	130

Table 3. *Relation between PFGE types and age of cattle*

	No. (%) of isolates						
Age of cattle	A	B	C	D	E	F	Total
<12 months	18 (16·7)	47 (43·5)	8 (7·4)	9 (8·3)	26 (24·1)	0 (0·0)	108
>12 months	0 (0·0)	6 (27·2)	0 (0·0)	0 (0·0)	0 (0·0)	16 (72·7)	22
Total	18	53	8	9	26	16	130

isolates of clusters D–F have increased since 1994 (Table 2).

Respecting the PFGE cluster and the age of cattle, 22 of 130 isolates were from adult (>12 months age), and, in these, 16 (72·7%) were cluster F and six (27·2%) were cluster B. Conversely, all isolates belonging to cluster F were from adult cattle (Table 3).

Comparative analyses of PFGE and RAPD profiles

Comparative data for PFGE and RAPD analyses for *M. haemolytica* isolates is presented in Table 1. All of the 79 isolates grouped into PFGE clusters A, B or C belonged to RAPD type I. Each of the nine of the PFGE cluster D isolates were consistent with RAPD type II, PFGE cluster E were RAPD type III and PFGE cluster F were RAPD type IV (Table 1, Fig. 2*b*).

DISCUSSION

It has been reported in several studies that *M. haemolytica* serotype A1 is often isolated as a causative agent from feedlot cattle with bovine respiratory disease [1, 20–22]. To elucidate transmissible routes of this disease, more highly discriminatory typing methods that can divide the identical serotype are necessary. In this report, we have applied two molecular typing methods (RAPD and PFGE) to distinguish genetic polymorphisms among *M. haemolytica* serotype A1 isolates in Japan.

The RAPD with primer PB-1 gave four distinct patterns (types I–IV) and among 130 isolates 79 isolates (60·3%) from 46 herds (63·3%) were classified into type I (Table 1). By PFGE analysis 130 isolates were divided into 15 PFGE profiles and six clusters (A–F) with a similarity of 62%. Comparing the two molecular methods, we found PFGE is more discriminative technique for typing *M. haemolytica* A1 than the RAPD. Even though the RAPD analysis is a quick, simple, less costly and less laborious, it was less discriminative than typing by PFGE. As a conventional PFGE protocol is laborious and so time consuming and it may take up to 7 days to produce results [23], in the present study, we applied modified rapid PFGE procedure that yields results in 2 days [17, 24].

We associated the PFGE clusters with the year of isolation and the geographical origins. Before 1994 only one isolate was classified into cluster F, and the other were grouped into clusters A, B or C. All isolates in cluster C were collected in the same prefecture until 1989 (Table 2). The numbers of isolates belonging to clusters D–F have increased since 1994, and since 1997 half of the isolates belong to these groups. These results show that strains of cluster A or B have existed in Japan since 1989, whereas strains of clusters D and E seem to have spread since 1994.

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Although the samples were collected from Hokkaido, the northernmost district in Japan, to Kyushu, the southern, so that there may be no regional dispersion, there was no relationship between molecular subtypes and geographic origin. The most prevalent PFGE subtype was B5, and was found in all of district investigated in this study. These results suggest that up to now, *M. haemolytica* serotype A1 strain with various molecular profiles have already spread in Japan and may have caused sporadic infections. It is possible reasons for lack of correlation between geographical distribution and molecular subtypes, that cattle are comparatively frequently transferred between prefectures.

The PFGE profile of cluster F was different from that of other clusters. In the PFGE profiles of cluster F, eight fragments were admitted and four of them were unique, which mean that cluster F isolates have specific characteristics. All isolates in the cluster F were isolated from adult cattle irrespectively of geographical origin and the year of isolation, therefore, there may be a correlation between pathogenesis of the strain and the host age and/or resistance.

It has been reported that *M. haemolytica* possesses many kind of virulence factors, which include a ruminant-specific leukotoxin, an anti-phagocytic capsule, lipopolysaccharide, iron-regulated outer membrane proteins, lipoproteins, a sialoglycoprotease, a neuraminidase and two potential immunoglobulin proteases [25–28]. In this investigation, we classified 130 isolates into four RAPD types and six PFGE clusters including 15 subtypes. The diversity among clusters may correspond to presence of these virulence factors. Further comparative analyses among clusters with identification of virulence factors are necessary.

In conclusion, RAPD typing and PFGE profiling, especially PFGE, are useful tools for epidemiological investigation of the identical serotype of *M. haemolytica*.

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