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Molecular Epidemiology of Japanese Avian *Pasteurella multocida* Strains by the Single-Enzyme Amplified Fragment Length Polymorphism and Pulsed-Field Gel Electrophoresis

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ABSTRACT. Molecular epidemiology analyses of the 36 clinical isolates of *Pasteurella multocida* from various avian hosts in Japan between 1976 to 2007 including 5 reference strains from the U.S.A., Taiwan and Indonesia were performed by employing the single-enzyme amplified fragment length polymorphism (SE-AFLP) comparison with the classical *Apa*I-based pulsed-field gel electrophoresis (PFGE). As the results, SE-AFLP gave 21 profiles while PFGE gave 20 profiles. The Simpson's index of diversity analysis indicated that SE-AFLP gave a high discrimination power than PFGE. This concluded that SE-AFLP is a higher discrimination power than PFGE to differentiate avian *P. multocida* isolates in Japan. In addition, the genetical profiles suggested that there is the evolution of somatic serotype 3 strain in the indigenous host of Japan.

KEY WORDS: discrimination power, *Pasteurella multocida*, PFGE, SE-AFLP.

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Fowl cholera is an important disease in poultry industry around the world. *Pasteurella multocida* of the gram negative bacteria is recognized as the etiologic agent of fowl cholera [16, 18]. The bacterium can be classified by capsular serogrouping into 5 serogroups (A, B, D, E and F) [6, 17] and by lipopolysaccharide into 16 somatic serotypes (1–16) [5]. Fowl cholera usually appears a septicemic disease in the acute or peracute conditions associated with the high mortality and morbidity but the chronic conditions are also observed [16, 18]. In Japan, strain of *P. multocida* capsular serogroup A and somatic serotype 3 was predominated among avian isolates and strains of somatic serotypes 1, 4 and 10 were also isolated [20]. Some of the clinical isolates were determined for their capsulation and virulence for chicken by the observation of their colonies by electron microscope or obliquely-transmitted light stereomicroscope and experimental infection in chickens [4]. Our previous investigations indicated the genetic variation of the adhesive protein gene among each isolates by PCR-RFLP employing the *Hind*III and *Eco*RI digestion [21]. However, the genetic epidemiology of these isolates needs to be clarified.

Since a number of DNA-based typing systems have been employed to characterize the isolates [1, 3, 10, 14, 15]. Pulsed-field gel electrophoresis (PFGE) is recognized as the classical DNA-based typing method of the isolates. However, the higher discrimination power DNA-based typing methods have been demonstrated. Previous studies suggested that the amplified fragment length polymorphism (AFLP) have been recognized as the typing method that showed the higher power of discrimination than either PFGE or the random amplified polymorphism DNA (RAPD) [1, 8]. However, the DNA-based typing method requires more knowledge about DNA to analyze [8]. Then, the recent study employed the SE-AFLP and PFGE to differentiate avian *P. multocida* isolates in Japan and the discriminatory power of the typing methods was also compared.

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MATERIALS AND METHODS

Bacterial strains and growth condition: *P. multocida* strains were supplied from the collection at the Laboratory of Veterinary Microbiology, Nippon Veterinary and Life Science University. Bacteria were cultured on dextrose starch agar (DSA; Difco Laboratories, MD) at 37°C for 18 hr. The morphology of the colony was observed with an obliquely-transmitted light stereomicroscope. Capsular typing was performed by employing the indirect hemagglutination (IHA) test [19] or multiplex PCR capsular grouping [22] and somatic serotyping was performed by the gel immunodiffusion (GID) test [7] as described previously. Single colony was picked up and continually cultured in tryptose broth (TB; Difco) at 37°C for 18 hr. Bacterial strains used in this study are shown in Table 1.

Isolation of DNA: Genomic DNA of the strains was prepared from 18-hr bacterial culture in TB by CTAB precipitation method as described previously [2]. DNA pellets were resuspended with TE buffer (pH 8.0) and kept at –20°C until used.

Single-enzyme amplified fragment length polymorphism (SE-AFLP): SE-AFLP was performed as described previously [12, 13]. Briefly, genomic DNA of the strains (6 μg)

Table 1. List of Japanese clinical *P. multocida* isolates and reference strains

Strain	Serovar	Origin	Place (Month,Year)	SE-AFLP profile	PFGE profile
Pm-1	A:3	Wild duck	Gunma (March, 1983)	14	18
Pm-3	A:3	Green pheasant	Saitama (February, 1984)	12	16
Pm-219	A:3	Green pheasant	Nagano (November, 1984)	15	19
Pm-448	A:3	Green pheasant	Fukushima (August, 1986)	13	17
Pm-441	A:3	Copper pheasant	Fukushima (August, 1985)	16	18
Pm-334	A:3	Chicken (Broiler)	Miyagi (May, 1985)	7	12
Pm-440	A:3	Chicken (Layer)	Nagasaki (June, 1983)	6	8
Pm-18	A:1	Myna bird	Fukuoka (May, 1976)	3	11
Pm-454	A:1	Goose	Aomori (March, 1987)	3	13
Pm-439	A:1	Chicken (Layer)	Nagasaki (June, 1983)	11	15
Pm-232	A:10	Chicken (Layer)	Fukuoka (May, 1980)	8	3
Pm-330	A:3,4	Chicken (Layer)	Kanagawa (May, 1985)	8	5
Pm-001	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-002	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-003	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-004	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-005	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-006	A:3	Chicken (Layer)	Saitama (August, 2003)	8	7
Pm-327	A:3,4	Chicken (Layer)	Kanagawa (May, 1985)	10	2
Pm-007	A:3	Chicken (Layer)	Niigata (August, 2005)	4	7
Pm-008	A:3	Chicken (Layer)	Niigata (August, 2005)	4	7
Pm-009	A:3	Chicken (Layer)	Niigata (August, 2005)	4	7
Pm-010	A:3	Chicken (Layer)	Niigata (August, 2005)	4	7
Pm-17	A:3	Chicken (Broiler)	Mie (October, 1980)	10	1
Oka-A	A:3	Green pheasant	Okayama (June-August, 2006)	4	3
Oka-B	A:3	Green pheasant	Okayama (June-August, 2006)	4	4
Oka-C	A:3	Green pheasant	Okayama (June-August, 2006)	9	3
KGS-1	B:2,3	Chicken (Broiler)	Kagoshima (August, 2006)	1	6
KGS-2	B:2,3	Chicken (Broiler)	Kagoshima (August, 2006)	1	6
KGS-3	B:2,3	Chicken (Broiler)	Kagoshima (August, 2006)	1	6
KGS-4	B:2,3	Chicken (Broiler)	Kagoshima (August, 2006)	1	6
Pm-011	A:3	Chicken (Layer)	Niigata (January-February, 2007)	5	7
Pm-012	A:3	Chicken (Layer)	Niigata (January-February, 2007)	4	7
Pm-013	A:3	Chicken (Layer)	Niigata (January-February, 2007)	4	7
Pm-014	A:3	Chicken (Layer)	Niigata (January-February, 2007)	4	7
Pm-015	A:3	Chicken (Layer)	Niigata (January-February, 2007)	4	7
X-73	A:1	Chicken	Reference strain (serovar A:1)	21	14
P-1059	A:3	Turkey	Reference strain (serovar A:3)	20	7
P-2100	A:10	Turkey	Reference strain (serovar A:10)	17	9
Bali	A:1	Chicken	Indonesia	18	20
Taiwan	A:1	Chicken	Taiwan	19	10

was digested with 24 U of *Hind*III (Takara, Shiga, Japan) at 37°C for 6 hr. Digested DNA was purified and 5 μl was taken and ligated with 0.2 μg of each ADH1 and ADH2 adapter oligonucleotides by 1 U of T4 DNA Ligase (Promega, Madison, WI, U.S.A.) at 22°C for 3 hr. Ligation mixture was heated at 80°C for 10 min and diluted 5 times with sterile distilled water and used as DNA template for PCR. PCR was performed with 5 μl of diluted ligation mixture, 2.5 mM dNTPs, 10 × PCR buffer, 2.5 mM $MgCl_2$, 300 *n*g of primer (HIA, HIT, HIC, HIG) and 1.25 U *rTaq* DNA polymerase (Takara). A cycle started at 94°C for 4 min and followed by 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2.5 min, then 1 cycle at 72°C for 7 min. PCR products were analyzed by 2.0% agarose gel electrophoresis in TBE buffer at 75 V. SE-AFLP patterns were observed by staining gel with ethidium bromide and photographed under UV illuminator (ATTO, Tokyo, Japan).

Pulsed-field gel electrophoresis: Pulsed-field gel electrophoresis was modified from the previously described method [11]. Briefly, bacteria were grown in TB broth at 37°C until OD_{600} 0.1 (approximately 1×10^8 cfu/m*l*). Bacterial suspensions were transferred to microcentrifuge tube and boiled for 30 sec. Then, bacterial cells were pelleted by centrifugation at 15,000 × g for 10 min. The cell were resuspended with EET buffer (100 mM EDTA, 10 mM EGAT, 10 mM Tris, pH 8.0) and gel plug was prepared by mixing with 1.6% chromosomal grade agarose (Bio-Rad, Foster city, CA, U.S.A.) in 0.5 × TBE buffer. Solidified suspensions were digested in EET-LS buffer (EET buffer, 200 μg/m*l* lysozyme, 0.05% sarcosyl) and incubated at 37°C for 6 hr. Then, plugs were digested in EET-SP buffer (EET buffer, 1 mg/m*l* proteinase K, 1% SDS) and incubated at 50°C for 24 hr. Subsequently, gel plugs were washed extensively 3 times with TE buffer (10 mM Tris [pH 8.0], 1 mM

EDTA) before equilibrated in 1 × L buffer (Takara) at room temperature for 15 min and then digested with 24 U *Apa*I (Takara) in 1 × L buffer and incubated at 37°C for 24 hr. Gel plugs were then subjected to load onto 1% pulsed-field certified agarose (Bio-Rad) in 0.5 × TBE buffer. Electrophoresis was carried out on the Bio-Rad CHEF II system (Bio-Rad) using 0.5 × TBE buffer with cooling system at 16°C during operation and electrophoresis at 6.0 V/cm with the initial switch time of 1 s, increasing to 40 s at 20 hr. Gels were stained and recorded as the photograph under UV illuminator (ATTO).

The discriminatory index: The discriminatory power (*D* value) of the typing methods was calculated by the Simpson's index of diversity as described previously [9].

Strain clustering: The relationship between *P. multocida* isolates were analyzed by the Bionumerics® version 4.0 (Applied Maths BVBA, Belgium).

RESULTS

Bacteria, capsular serogroup and somatic serotype: Total of 36 strains were isolated from clinical cases of different hosts including 5 reference strains (Table 1). Most of the isolates were classified into capsular serogroup A except for four isolates (KGS1-4), which were isolated from the case of chronic purulent arthritis and polyserositis in layers, were classified into capsular serogroup B. Somatic serotype 3 strains were predominated among avian clinical isolates and somatic serotype 1 (3 strains), 10 (1 strain), shared somatic types 3 and 4 (2 strains) and shared somatic serotypes 2 and 3 (4 strains) were also obtained.

PFGE analysis: Digestion with the *Hpa*II showed the 20 profiles among the 36 avian isolates including 5 reference strains (Table 1). Profile type 7 was the most prevalent and consisted of only somatic serotype 3 isolates including strain P-1059 (serovar A:3). Strain OKA-A and OKA-C were classified into the same profile type (type 3) but only OKA-B showed its own type (type 4). The PFGE profile of strains KGS1–4 (serovar B:2,3) were separated and classified in profile type 6. Strains Pm-18 (type 11), Pm-454 (type 13) and Pm-439 (type 15) showed their own profile types that differed from strain X-73 (serovar A:1, type 14). Strain Pm-232 (serovar A:10) was classified into profile type 3 which differed from strain P-2100 (serovar A:10, profile type 9). Strains Pm-232 and Pm-330 (serovar A:3,4) were classified into profile types 2 and 5, respectively. PFGE-based phylogenetically diagram is shown in Fig. 1. There was closely relationship among Japanese somatic serotype 3 isolates and strain P-1059 from U.S.A. As observed, strains X-73 (A:1) and P-2100 (A:10) locate in their own cluster while those serovars of Japanese isolates located in the same cluster with all the isolates. Strain Pm-454 (goose) locate at the same cluster with serovar A:1 isolate from Taiwan's chicken while strain Pm-18 (myna bird) locate at the same cluster with Indonesian chicken isolate. Moreover, only one isolate of serovar A:1 (Pm-439) and one isolate of serovar A:10 (Pm-232) locate at the same cluster with serovar A:3 isolates.

SE-AFLP analysis: Using the SE-AFLP, all 36 isolates and 5 reference strains could be classified into 21 profiles (Table 1), and the cluster analysis was shown in Fig. 2. Most isolates showed their own profile by SE-AFLP typing system. Profile types 4 and 8 were the most prevalent of serovar A:3 isolates including strains Pm-232 (serovar A:10) and Pm-330 (serovar A:3,4), while strain Pm-327 (serovar A:3,4) was classified into profile type 10 together with strain Pm-17 (serovar 3). SE-AFLP profile of strains KGS1-4 (serovar B:2,3) was classified into their own profile type (type 1). All 3 reference strains were classified into their own profile type that separated from all the Japanese avian isolates. The SE-AFLP-based phylogenetically diagram is shown in Fig. 2. Strain of Japanese serovar A:1 locate at the same cluster but differ from strains X-73, Taiwan or Bali. Moreover, strain of Japanese serovar A:10 locate at the same cluster with serovar A:3 isolates but different cluster to strain P-2100.

The index of discrimination (D value): The application of Simpson's index of diversity was performed with formulation as described. The *D* value of SE-AFLP was 0.904 while the *D* value of PFGE was 0.841. A comparison *D* value among two typing methods indicated that the SE-AFLP gave a high power to differentiate the isolates than PFGE typing method.

Making of dendrograms: The Pearson correlation for analyzing the similarity coefficient and UPGMA for making the dendrogram type were performed and showed the dendrogram in Figs. 1 (PFGE) and 2 (SE-AFLP).

DISCUSSION

Since *P. multocida* was recognized as the etiologic agent of fowl cholera, capsular serogroup A and somatic type 1, 3 or 4 have been predominated in serovars of clinical isolates around the world. In Japan, there was no report of outbreak for more than 20 years until 1976. The outbreak occurred in myna bird imported from Thailand. Since that time, the outbreaks in various hosts have been reported. Capsular serogroup A and somatic serotype 3 (A:3) was predominated serovar and the some of these isolates produced the lower amount of capsule material [4, 20]. A non-capsulated strain of *P. multocida* was obtained from strain P-1059 (serovar A:3) by 35-serial passages on DSA plates and designated strain P-1059B [4]. This variant strain produced bluish colony on DSA observed by the obliquely-transmitted light stereomicroscope and its capsule thickness also significantly related to its pathogenicity for chickens (Borrathybay *et al.*, 2003). Our preliminary investigation on avian pasteurellosis had identified the gene coding of the adhesive protein (*cp39*) of the avian isolates and the PCR-RFLP employed *Eco*RI or *Hind*III was performed [21]. The PCR-RFLP indicated that 2 strains of somatic type 3 and 4 strains of somatic type 1 strain could not be digested with the *Eco*RI or *Hind*III. The genetic variations among the adhesive protein gene of the isolates were observed and required more expla-

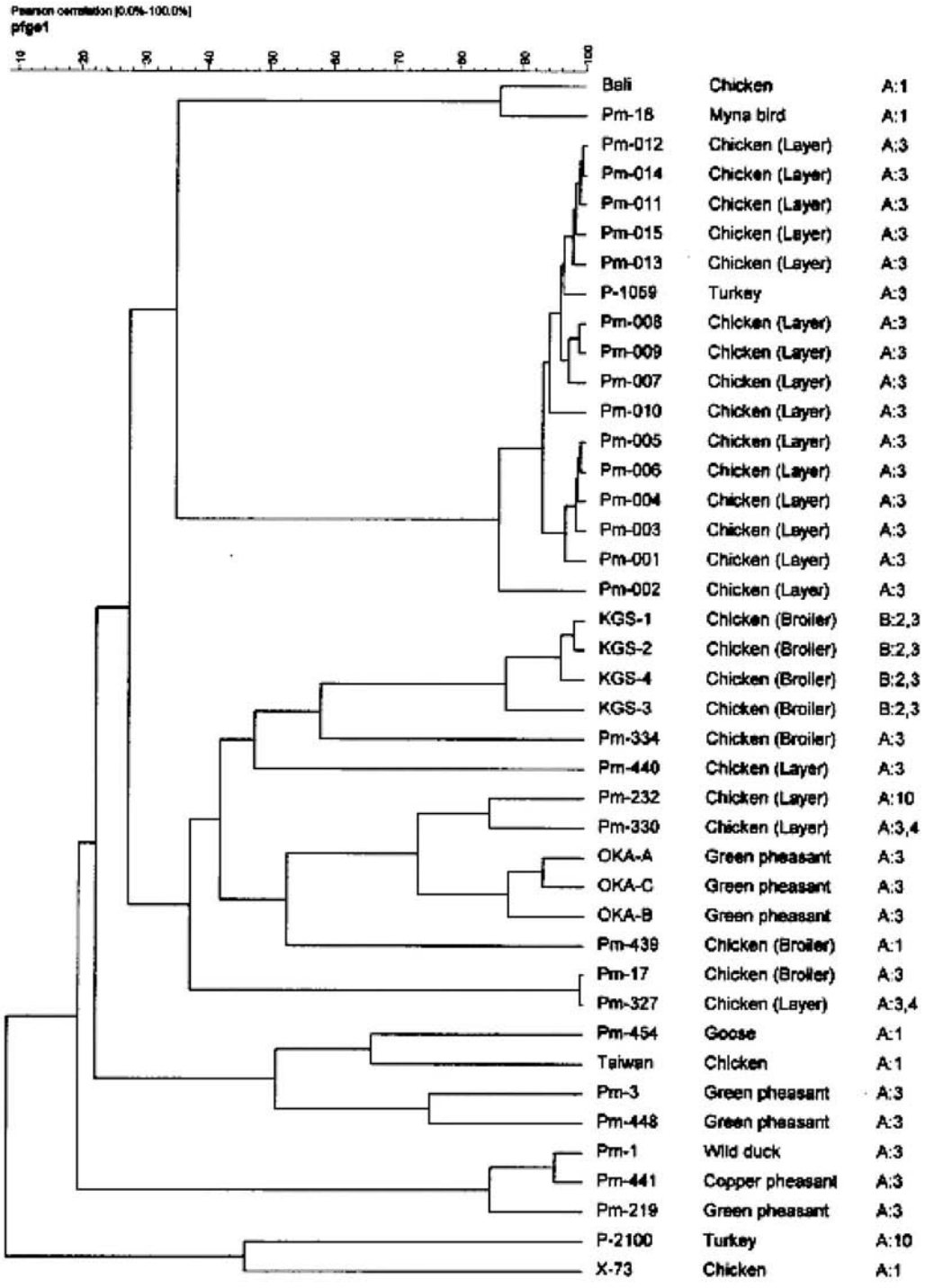

Fig. 1. Pulsed-field gel electrophoresis-based cluster of *P. multocida* strains. Phenogram was created by Pearson correlation for analyzing the similarity coefficient and UPGMA for making the dendrogram type ($P<0.05$).

nations.

The genetical techniques have been applied to differentiate epidemiologically significant strains of *P. multocida* such as ribotyping [15], multilocus enzyme electrophoresis [3], RAPD [8, 11], AFLP [1, 13] or PFGE [11]. The PFGE was recognized as the gold epidemiological method for differentiation of isolates while the advantages of the SE-AFLP were much more stringent, consistent, reproducible and easy to optimize the method than RAPD method [8]. As our present results, the SE-AFLP (0.904) had more power of

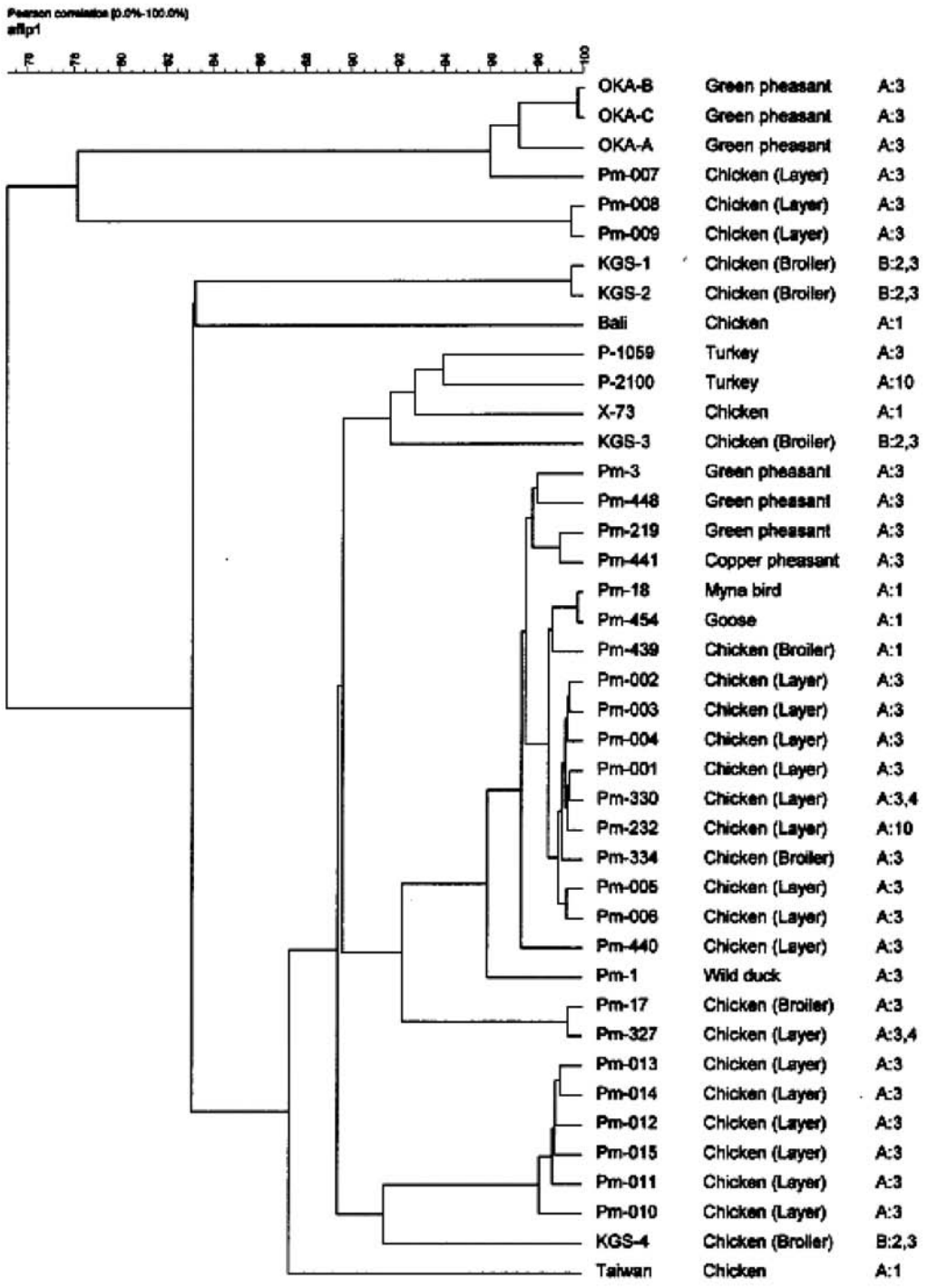

Fig. 2. Single enzyme-amplified fragment length polymorphism-based cluster of *P. multocida* strains. Phenogram was created by Pearson correlation for analyzing the similarity coefficient and UPGMA for making the dendrogram type ($P<0.05$).

discrimination than PFGE (0.841). This indicated that SE-AFLP is suitable for the rapid fingerprinting of the isolates as suggested in the previous works [13]. In additions, SE-AFLP is less time consuming method than PFGE but the disadvantage of SE-AFLP is difficulty in clarifying the appropriate primers and conditions.

The Japanese avian isolates were classified into their own cluster and differed from their 5 reference strains. Isolates

before year 2003 showed their own and there was no relation between character of the strain and the location of outbreaks or hosts. In addition, these isolates have been isolated from the indigenous birds such as green pheasants and copper pheasants. In contrast, the isolates after year 2003 showed the group characteristics. Interestingly, all of the isolates showed their own SE-AFLP or PFGE profiles and were different from the reference strains. The previous reports indicated that the green pheasants were highly susceptible to the isolates as compared to chickens [20]. In Japan, the pheasants were raised in the net and will be released into nature for hunting. The outbreaks in pheasants possibly occur by transmission of bacteria through the healthy carrier birds released in the nature. This suggestion was supported by the isolations of somatic serotype 3 strain from the green pheasant flocks in 2006. The isolates derived from host, particularly from poultry since year 2003, belonged to somatic serotype 3. These observations coupled with the SE-AFLP and PFGE profiles indicate that there is the evolution of somatic serotype 3 strain in the indigenous hosts of Japan.

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