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Full Length Research Paper

Comparison of *ompP5* sequence-based typing and pulsed-filed gel electrophoresis for genotyping of *Haemophilus parasuis*

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In this study, comparison of the outer membrane protein P5 gene (*ompP5*) sequence-based typing with pulsed-field gel electrophoresis (PFGE) for the genotyping of *Haemophilus parasuis*, the 15 serovar reference strains and 43 isolates were investigated. When comparing the two methods, 31 *ompP5* sequence types (STs) and 43 PFGE types were identified with *ompP5* STs A16 and A28 being the dominant *ompP5* ST types, while no predominant PFGE type was found. The discriminatory indices were 0.95 for *ompP5* sequence-based method and 0.98 for PFGE, and the two techniques were proved to be 70.7% congruent. Therefore, *ompP5* sequence-based typing was a simple and inexpensive method, enabling it as a preliminary technique to research the molecular differentiation of *H. parasuis*.

Key words: *Haemophilus parasuis*, *ompP5*-based typing method, PFGE, molecular typing.

INTRODUCTION

Haemophilus parasuis is the etiological agent of Glässer's disease in pigs, which is characterized by fibrinous polyserositis, polyarthritis and meningitis (Oliveira and Pijoan, 2004). Genetic and phenotypic variation has been observed among *H. parasuis* strains, while 15 serovars of *H. parasuis* have been described (Olvera et al., 2006a; Kielstein and Rapp-Gabrielson, 1992). However, a high percentage of strains are non-typeable by serotyping, which would indicate the possibility that a larger number of serovars require more detailed characterization. Therefore, the availability of convenient, rapid and universal typing tools for the molecular characterization of *H. parasuis* becomes a high priority.

Currently, several groups have developed various genotyping methods and attempted to achieve a more precise diversity analysis of *H. parasuis* strains. The majority of these groups focused on fingerprinting methods (Blackall et al., 1997; del Rio et al., 2006; Rafiee et al., 2000), which were fast, inexpensive and allowed the confirmation of the number of strains involved in an outbreak. Moreover, the fingerprinting methods also achieved a higher level of discrimination than serotyping, but none has been standardized across laboratories (Olvera et al., 2007). Pulsed-field gel electrophoresis (PFGE) is described as one of the most powerful microbial genotyping methods and is accepted as a “gold standard” for typing bacteria (Huang et al., 2005), which has been applied also in H. parasuis (Zhang et al., 2011). The H. parasuis ompP5 gene, encoding outer membrane protein P5 (OmpP5), exhibits a degree of sequence polymorphism and specific structure, which makes it a potential molecular marker for genotyping of H. parasuis (Mullins et al., 2009; Tang et al., 2010).

The objective of this work was to compare the capability of ompP5 sequence-based typing and PFGE for genotyping analysis of 15 H. parasuis serovar reference strains and 43 clinical isolates. The results indicate that ompP5-based typing served as a simple and comparably precise molecular method, which could provide preliminary data for molecular differentiation of *H. parasuis*.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

The 15 *H. parasuis* serovar reference strains were kindly supplied by the Huazhong Agricultural University of China. The 43 clinical strains were isolated from 38 farms in Southern China between 2008 and 2010 from cases of meningitis, arthritis or pneumonia in pigs with suspected Glässer's disease, and the isolates were serotyped using gel diffusion and indirect haemagglutination tests (Kielstein and Rapp-Gabrielson, 1992; Turni and Blackall, 2005) (Table 1). *H. parasuis* strains were cultivated in Trypticase Soy Agar (Oxoid, UK) supplemented with 0.002% nicotinomide adenine dinucleotide (Sigma, USA) and 5% inactivated bovine serum at 37°C in a 5% CO_2-enriched atmosphere.

Polymerase chain reaction (PCR), sequencing and analysis

The amplification of the *ompP5* gene was performed by using the following primers: forward 5'-GCATTCTTGCCTCGTTCTTT-3'; reverse 5'-CCGGTGAAGAAATAGA TGGG-3', as described previously (Tang et al., 2010). The PCR was performed using a Platinum *Pfx* DNA polymerase kit (Invitrogen, USA) according to the manufacturer's instruction. The *ompP5* gene's amplicons were cloned into pMD-19T vector (TaKaRa, China) and sequenced by the automated DNA sequencing system of an Applied Biosystems model 3730 (Applied Biosystems, USA), which were performed according to Tang et al. (2010).

Alignment of *ompP5* gene was performed with the Clustal X alignment in MegAlign (Lasergene Inc. USA). Secondary structure prediction was performed by the Psipred secondary structure prediction method (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). A sequence type (ST) was defined by comparing every different sequence of *ompP5* gene and giving each different sequence a ST number, even if only one nucleotide was different. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1.

PFGE

PFGE was performed as described previously (Zhang et al., 2011). Briefly, the agarose-embedded *H. parasuis* genome was digested with 50 U of *Cpo*I (TaKaRa, Japan) in a water bath at 30°C for 2 h. The restriction fragments were separated by electrophoresis in 0.5 × Tris-borate- ethylene diamine tetra-acetic acid (EDTA) buffer at 14°C for 21 h using a Chef Mapper electrophoresis system (Bio-Rad, USA) with pulse times of 2.16 to 63.8 s. The gels were stained with ethidium bromide, and the DNA bands were visualized by UV transillumination (Bio-Rad, USA). *Salmonella enterica* serovar Braebderup H9812 was used as the control strain, which was digested with 50 U of XbaI (TaKaRa, Japan). Isolates presenting DNA smear patterns were retested.

An interpretation of DNA fingerprint patterns was accomplished using Bionumerics 4.0 software (Applied Maths, USA). Based on the PFGE criteria for bacterial strain typing [8], isolates with indistinguishable restriction patterns were defined as a single PFGE type. The discriminatory index (DI) of both typing methods was determined by the application of Simpson's index (Hunter and Gaston, 1988). The method was calculated according to the following formula:

$$DI = 1 - \left(\sum n(n-1) \Big/ N(N-1) \right)$$

where, DI is the diversity, N is the total number of strains in the sample, and n is the number of strains in each type.

The indicator function of $C = \frac{\sum_{i=1}^{n} I_i}{N}$ was applied to calculate the congruence between the *ompP5* sequence and PFGE, where C is the congruence index, N is the total number of strains in the sample, and

$$I_i = \begin{cases} 1, \text{ when there is congruence in N category.} \\ 0, \text{ otherwise.} \end{cases} \quad (i = 1, 2, ..., n.)$$

RESULTS AND DISCUSSION

OmpP5 sequence-based typing of *H. parasuis*

Among all of the strains, there were *ompP5* ORFs of five different lengths: 1098bp, 1104bp, 1101bp, 1110bp and 1116bp (GenBank accession numbers: HM747071-HM747113, FJ667983-FJ667995, EU846096 and EU846097). The length of 1,101bp was first identified in this study, and the novel of *ompP5* allele was distinguished by nucleotides insertion occurred in predicted surface-exposed loop region of the OmpP5. This phenomenon was possibly due to the different host immune selection and high rates of recombination that occurred in *H. parasuis* (Olvera et al., 2006a, b). An alignment of *ompP5* gene sequences using MegAlign indicated 86.3 to 100% similarity among the 58 *H. parasuis* strains. The diversity of the nucleotide sites was marked by unbalanced variation rates, which were mainly the located sites of variation in predicted surface-exposed loops regions of the OmpP5 protein that have been performed as previously reported (Mullins et al., 2009; Tang et al., 2010). The nucleic acid coding sequence for *ompP5* was used to construct a neighbor-joining dendrogram, and two major evolutionary lineages were defined (Figure 1).

The method of ST has been used as a genotyping method in epidemiological investigations of bacterial species (Huang et al., 2005; Olvera et al., 2006a; Tang et al., 2010). In this study, 31 different *ompP5* STs were identified from the 58 *H. parasuis* strains (indicated by A1 to A31) (Table 1). The 15 reference strains were assigned to 12 different ST types, and the 43 clinical isolates were clustered to 20 different *ompP5* ST types among which ST A1 (8/43), and A26 (10/43) were identified as the two most common STs. From serovars of isolates, nine predominant serovar 5 isolates were assigned as ST A26 (6/9), ST A11 (1/9), ST A15 (1/9) and ST A31 (1/9); nine predominant serovar 4 isolates were assigned as ST A1 (4/9), ST A14 (2/9), ST A12 (1/9), ST 25 (1/9) and ST A26 (1/9). The phenomenon indicated that *ompP5* ST results might be associated with the results of serotyping in isolates. Furthermore, five groups of isolates derived from the same farm shared the

Table 1. *Haemophilus parasuis* used in this study.

Strain (serovar)	Isolation site	Farm	*ompP5* ST	PFGE type	*ompP5* accession number	PFGE source
***H. parasuis* reference strains**						
N4(serovar 1)			A7	B17	FJ667983	Zhang et al., 2011
SW140(serovar 2)			A 3	B31	FJ667984	Zhang et al., 2011
SW114(serovar 3)			A 27	B16	FJ667985	Zhang et al., 2011
SW124 (serovar 4)			A 27	B16	EU846096	Zhang et al., 2011
Nagasaki(serovar 5)			A 16	B35	EU846097	Zhang et al., 2011
131 (serovar 6)			A 28	B42	FJ667986	Zhang et al., 2011
174(serovar 7)			A 3	B25	FJ667987	Zhang et al., 2011
C5(serovar 8)			A 28	B13	FJ667988	Zhang et al., 2011
D74(serovar 9)			A 29	B41	FJ667989	Zhang et al., 2011
H367(serovar 10)			A 19	B14	FJ667990	Zhang et al., 2011
H465(serovar 11)			A 8	B22	FJ667991	Zhang et al., 2011
H425(serovar 12)			A 10	B4	FJ667992	Zhang et al., 2011
IA-84-17975(serovar 13)			A 17	B24	FJ667993	Zhang et al., 2011
IA-84-22113(serovar 14)			A 22	B39	FJ667994	Zhang et al., 2011
SD-84-15995(serovar15)			A 20	B40	FJ667995	Zhang et al., 2011
***H. parasuis* field strain**						
SC091 (serovar 4)	Lung	1	A 1	B9	HM747071	Zhang et al., 2011
SC090 (serovar 10)	Synovia	2	A 1	B9	HM747073	This study
SC081 (serovar 4)	Lung	3	A 1	B9	HM747074	This study
SC054 (serovar 4)	Lung	4	A 1	B9	HM747075	This study
SC096 (serovar 4)	Synovia	5	A 1	B9	HM747072	This study
SC016 (serovar 2)	Lung	6	A 1	B28	HM747077	This study
SC020 (serovar15)	Heart	6	A 1	B28	HM747078	This study
SC001 (serovar15)	Lung	7	A 1	B28	HM747076	Zhang et al., 2011
SC080 (serovar 13)	Lung	8	A 2	B34	HM747080	This study
SC013 (serovar 2)	Synovia	9	A 2	B34	HM747079	This study
SC066 (serovar 6)	Synovia	10	A 4	B29	HM747081	This study
SC111 (serovar 12)	Lung	11	A 5	B27	HM747082	This study

Table 1. Contd.

Strain (serovar)	Isolation site	Farm	ompP5 ST	PFGE type	ompP5 accession number	PFGE source
SC044 (NT§)	Lung	12	A 6	B43	HM747083	This study
SC021 (NT)	Lung	13	A 9	B26	HM747084	This study
SC022 (NT)	Lung	13	A 9	B35	HM747085	This study
SC032 (serovar 5)	Synovia	14	A 11	B23	HM747086	This study
SC060 (serovar 4)	Synovia	15	A 12	B15	HM747087	Zhang et al., 2011
SC095 (serovar 11)	Lung	16	A 13	B12	HM747088	This study
SC067 (serovar 4)	Lung	17	A 14	B19	HM747089	Zhang et al., 2011
SC068 (serovar 4)	Lung	17	A 14	B20	HM747090	This study
SC083 (serovar 5)	Lung	18	A 15	B36	HM747091	This study
SC053 (NT)	Lung	19	A 16	B33	HM747092	This study
SC051 (serovar 6)	Lung	19	A 16	B32	HM747093	This study
SC033 (serovar15)	Synovia	20	A 18	B18	HM747094	This study
SC069 (serovar 7)	Lung	21	A 21	B10	HM747095	This study
SC110 (NT)	Lung	22	A 23	B2	HM747096	This study
SC028 (serovar 13)	Heart	23	A 24	B1	HM747097	This study
SC112 (serovar 2)	Lung	24	A 24	B37	HM747098	This study
SC077(serovar 4)	Nasal	25	A 25	B5	HM747099	Zhang et al., 2011
SC099 (serovar15)	Brain	26	A 26	B6	HM747101	This study
SC094 (serovar 5)	Nasal	27	A 26	B30	HM747103	Zhang et al., 2011
SC019 (NT)	Brain	28	A 26	B3	HM747104	Zhang et al., 2011
SC079 (serovar 5)	Nasal	29	A 26	B21	HM747107	Zhang et al., 2011
SC049 (serovar 5)	Lung	30	A 26	B6	HM747109	This study
SC057 (serovar 9)	Lung	31	A 26	B30	HM747108	This study
SC085 (serovar 4)	Lung	32	A 26	B38	HM747106	This study
SC043 (serovar 5)	Lung	33	A 26	B6	HM747105	This study
SC063 (serovar 5)	Heart	34	A 26	B38	HM747100	Zhang et al., 2011
SC042 (serovar 5)	Lung	35	A 26	B6	HM747102	Zhang et al., 2011
SC030 (serovar 13)	Lung	36	A 30	B11	HM747110	Zhang et al., 2011
SC025 (NT)	Lung	36	A 30	B11	HM747111	This study
SC070 (NT)	Lung	37	A 31	B7	HM747112	This study
SC089 (serovar 5)	Lung	38	A 31	B8	HM747113	This study

§, Non-typeable.

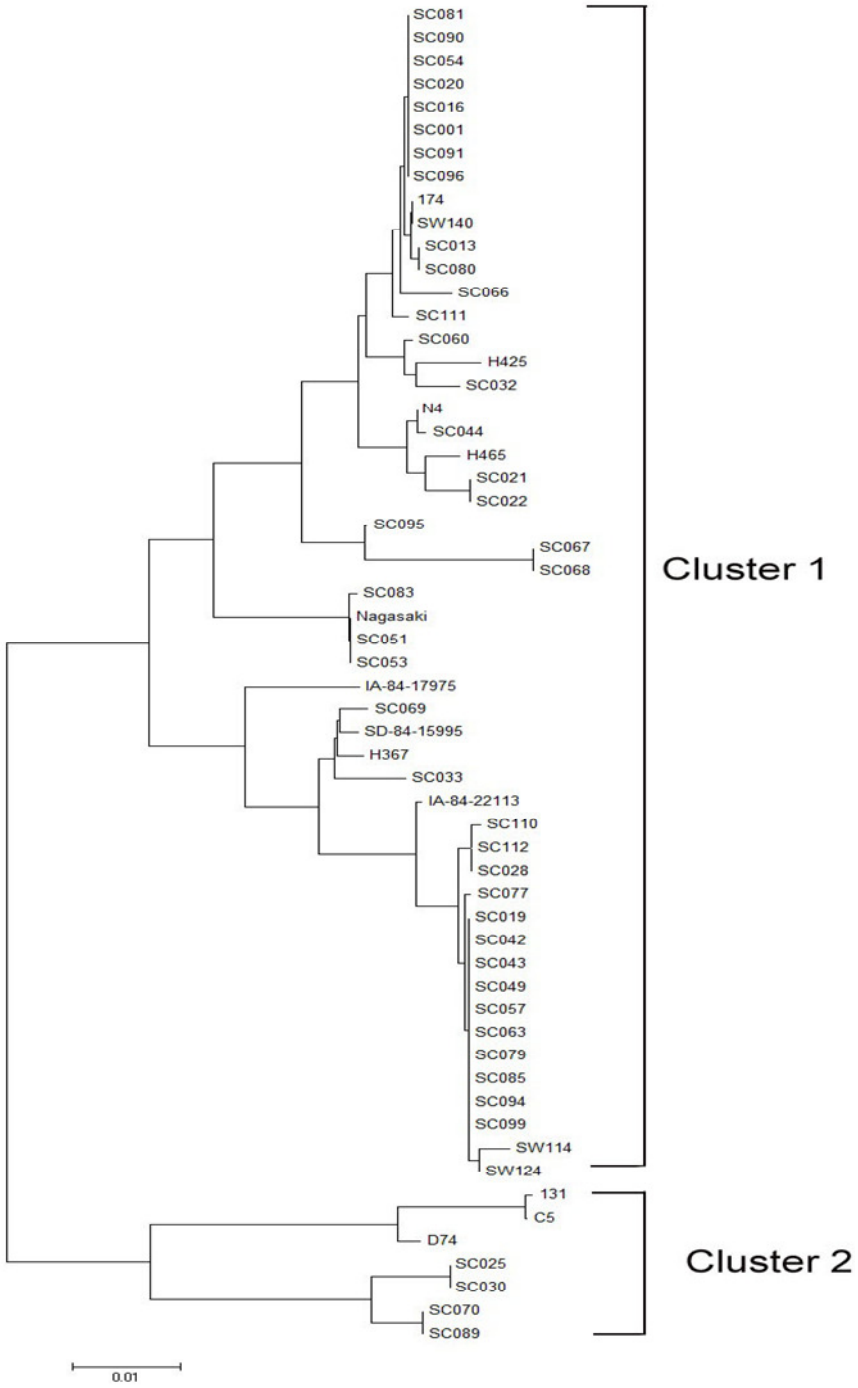

Figure 1. Phylogenetic consensus tree for the *Haemophilus parasuis ompP5* gene. The tree was constructed by the neighbor-joining method using MEGA 4.1 software (www.megasoftware.net).

same *ompP5* ST type (Table 1), which suggested that *ompP5* sequence-based typing method seemed to be advantageous in identifying clonally related isolates from the same farm. Of course, more clinical isolates from the same farm are needed to further confirm this phenomenon.

PFGE typing of *H. parasuis*

In this study, restriction digestion of *H. parasuis* chromosomes by *Cpo*I generated various PFGE types. 43 distinct PFGE types were observed among the 58 strains (indicated by B1 to B43) (Table1). Nine serovar 4 isolates

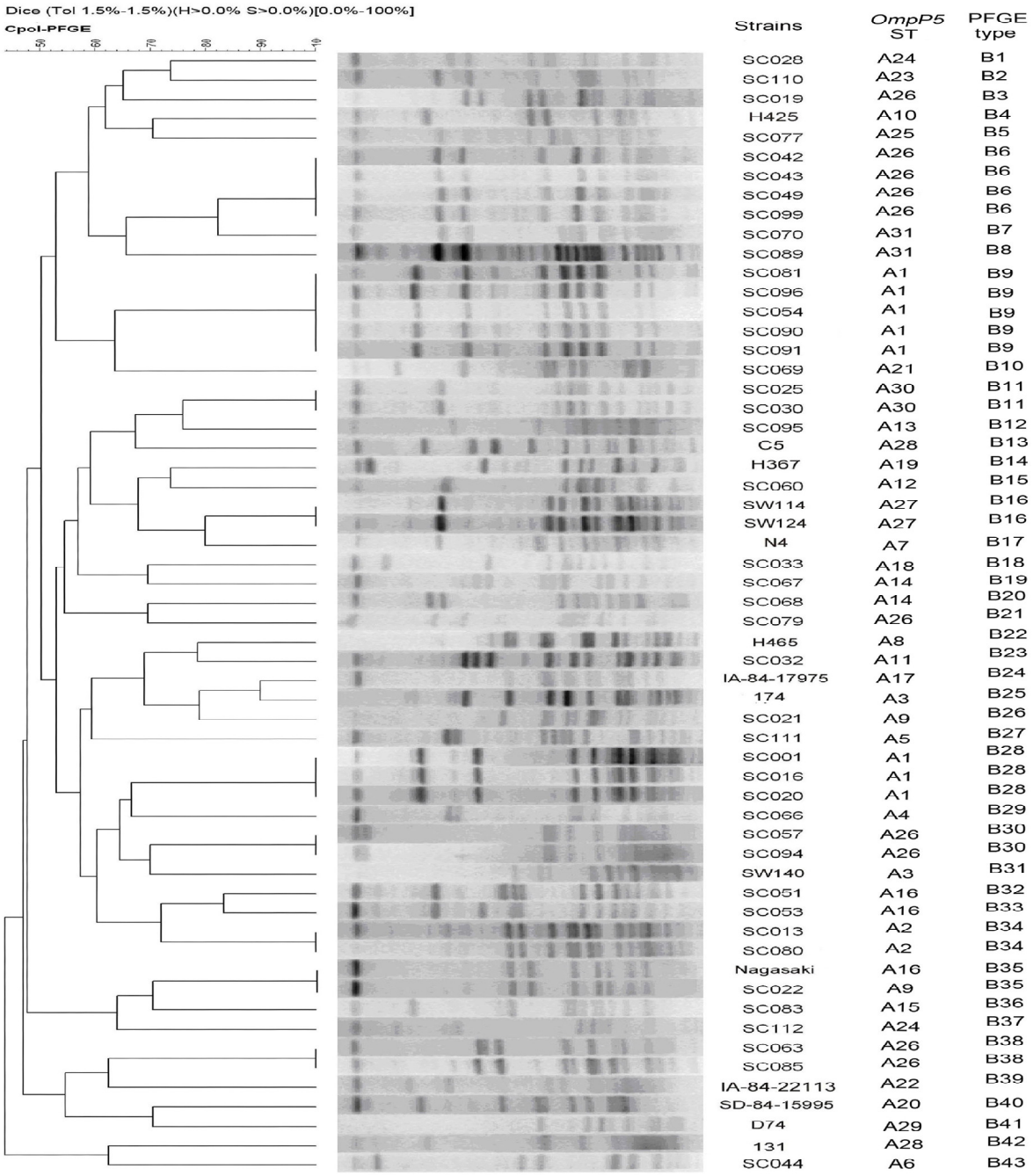

Figure 2. Dendrogram of PFGE fingerprints created using UPGMA clustering of Dice coefficient values. The similarity matrix was based on band-matching analysis; optimization and position tolerance settings were 1.00 and 1.5%, respectively.

were determined as six different PFGE types; nine serovar 5 isolates were identified as eight distinct PFGE types. No predominant PFGE type could be found in clinical isolates and no correlation between PFGE types and serovars was observed in this study. The phenomenon of high PFGE profile variability indicated a high genomic plasticity occurred in *H. parasuis*. The relatedness among the *H. parasuis* strains was evaluated by the Bionumerics 4.0 software (Applied Maths, USA) and was depicted in an unweighted pair-group method with arithmetic averages (UPGMA) dendrogram (Figure 2). No obvious association with virulence was observed. For example, the reference serovar 4 strain (virulent) and the reference serovar 3 strain (non-virulent) belonged to the same PFGE type. In addition, though isolates (SC016 and SC020, SC025 and SC030) from the same farm

were characterized as the same type, diverse PFGE types were occasionally assigned to isolates (SC021 and SC022, SC051 and SC053, SC067 and SC068) from the same farm, as well (Table 1). Due to the higher discriminatory power and no standard database available of PFGE in *H. parasuis*, hence, a phenomenon of identifying clonally related isolates from the same farm by PFGE was not fully described in PFGE typing in this present study.

Comparison of the two molecular typing methods

The discriminatory power calculated with Simpson's index of diversity was 0.95 for *ompP5* sequences typing, and 0.98 for PFGE, respectively. The higher the Simpson's index, the greater the possibility that two unrelated strains will be classified into different types. For both techniques calculated by indicator function, the best association was 70.7% among all 58 *H. parasuis*, 86.7% among reference strains, and 62.8% among clinical isolates. In *Campylobacter* strains, the *cmp* (encoding the surface-exposed major outer membrane) sequence types were 77.6% congruent with the PFGE types which suggested the feasibility of the *cmp*-based method (Huang et al., 2005). In *S. enterica*, the multiple locus variable number of tandem repeats typing method (MLVA) which detected polymorphisms at genomic loci based on PCR, showed 59.8% congruency with PFGE, indicating that it was a good alternative technique for epidemiological investigations (Davis et al., 2009). Therefore, we believed that the 70.7% congruency between the two techniques indicated that *ompP5*-based typing method showed good congruence with PFGE types, making it as a good preliminary technique to investigate the genotypes of *H. parasuis*.

As a sequence-based typing tool, there were several advantages to the *ompP5* gene. First, as a surface-exposed bacterial structural protein, OmpP5 protein showed considerable heterogeneity mainly in regions corresponding to predicted surface-exposed loops. Hence, this characteristic of OmpP5 protein made its gene sequence a potential molecular marker for genotyping in *H. parasuis* (Mullins et al., 2009; Tang et al., 2010). Secondly, the *ompP5* gene-encoding OmpP5 protein appeared to be an essential gene and is present in every *H. parasuis* strain tested in our laboratory. Finally, the two ends of the *ompP5* gene were highly conserved in *H. parasuis*, which facilitated the designing of PCR primers to amplify the gene in various strains of *H. parasuis*. Thus, the sequencing of *ompP5* was easy to compare and reproduce among different laboratories.

In conclusion, based on discrimination power, PFGE was more effective than *ompP5*-based typing method at genotyping *H. parasuis*. However, *ompP5*-based typing method had a good correlation with PFGE and serovar, therefore could be considered as a possible tool for genotyping the isolates.

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