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Genotyping of *Haemophilus Parasuis* Isolated from Northwest China Using PCR-RFLP Based on the *ompA* Gene

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ABSTRACT. Outer membrane proteins (OMPs) are the major virulent factors of *Haemophilus parasuis*. PCR-RFLP targeting the *ompA* gene was conducted to investigate the possibility of genotyping *H. parasuis* in this study. Fifteen reference strains and 49 isolates from pig farms in northwest China were genotyped by PCR-RFLP with a pair of specific primers. The results indicated that both the 15 reference strains and 49 isolates could be classified into 8 different genotypes by PCR-RFLP, respectively. Seven genotypes including AA, BB, BA, CA, BC, BD and CD existed simultaneously in the reference strains and isolates, but genotype CB only existed in the isolated strains. Interestingly, genotypes BA, CD and CA were only found in diseased pigs and accounted for 38.8%, 22.4% and 18.4% of the isolates, respectively. On the other hand, strains isolated from apparently healthy pigs were classified into genotypes AA, BB, BC and CB. However, the virulent reference serovar 1 strain has an AA genotype, and the fact that nearly all strains from the healthy pigs belonged to serovars classed as virulent suggests that these genotypes might also include virulent strains; therefore, further validation with more field strains is needed. The capability of the RFLP-PCR method based on the *ompA* gene for genotyping *H. parasuis* isolates indicates that this method may be a useful tool for epidemiological study.

KEY WORDS: genotyping, *Haemophilus parasuis, OmpA*, PCR-RFLP.

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Haemophilus parasuis (Hps) is the pathogen of Glässer's disease, which is characterized by fibrinous polyserositis, meningitis and arthritis in juvenile pigs [9]. *H. parasuis* is part of the normal respiratory microbiota and was previously thought to be an opportunistic pathogen affecting only health compromised pigs. However, reviews in the last years concluded that it is responsible for the major epidemic outbreaks among high health pig herds, causing high mortalities [8]. Glässer's disease is an important emerging infectious disease influencing the pig industry worldwide [7], including China [2, 5].

The traditional identification method of *H. parasuis* is isolation via culture and biochemical testing of the pathogen. *H. parasuis* can be divided into 15 serovars by a serological method [6]. Kielstain and Rapp-Gabrielson classed these 15 serovars into highly pathogenic, moderately pathogenic, mildly pathogenic and non-pathogenic. This classification has not held up for field isolates in recent years. A study by Aragon *et al.* has indicated that there is variability in the virulence of different strains of the same serovar suggesting that the serovar is not a good predictor of pathogenicity of field strains [1]. It is not known whether homologous protection is achieved against all strains within one serovar. The knowledge of cross-protection among different serovars is limited to a few studies [8]. In addition, 15–41% of clinical strains can't be typed by serological typing methods [10].

Advances in molecular biology and genotyping have contributed significantly to the typing of *H. parasuis*. Smart *et al.* identified the different *H. parasuis* strains using DNA-based restriction endonuclease pattern (REP) [13]. Rafiee *et al.* amplified the genotypes of different strains of *H. parasuis* by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and detected no close relation between virulence and genotypes [11]. Recently, Restriction Fragment Length Polymorphism (RFLP)-PCR was established for the genes of *tbpA*, *tbpB* and *aroA* of *H. parasuis*. The result of genotyping isolated strains of *H. parasuis* via RFLP-PCR showed that there was no significant correlation between the genotypes, virulence and serotypes of the *H. parasuis* isolates [3, 4]. Recent studies have shown that the outer membrane proteins (OMPs) may be related to the virulence of *H. parasuis* [12]. Using the PCR-FRLP technique, the current study compared 49 isolates from pig farms in northwest China and 15 reference strains of *H. parasuis* in regard to their genotypes, serovars and virulence based on the *ompA* gene.

MATERIALS AND METHODS

Medium: Tryptone soya agar (TSA) and tryptone soya broth (TSB) medium (Becton, Dickinson and Company) was used with the addition of a final concentration of 10% horse serum, 5% yeast extract and 0.05% NAD.

Bacterial strains: The 15 reference strains of *H. parasuis*

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were kindly provided by Dr Pat Blackall, Qld DPI, Animal Research Institute, Australia. The 49 field isolates originated from local pig farms in northwest China and were serotyped by the gel diffusion method of Kielstein *et al.* [6]. *Actinobacillus pleuropneumoniae* (App) representing 12 serovars were also used in this study.

DNA extraction: The reference and isolated strains of *H. parasuis* from healthy and sick pigs were inoculated onto TSA. After inoculation at 37°C for 24 hr, one selected colony was inoculated into TSB and cultured in a shaking bed at 37°C at 180 rpm for 11–14 hr. DNA was extracted from 1 m*l* of the culture (1–2 × 10^9 CFU) with a TIANamp Bacteria DNA Kit (Takara Sake U.S.A. Inc.) according to manufacturer's instructions.

PCR amplification: Primers for the *ompA* gene were designed with the Primer Premier 5.0 program (PREMIER Biosoft International) with reference to the *omp*A sequence (EU846097) of serotype 5 of *H. parasuis* in GenBank. The primers HpsF (5'-ATGAAAAAATCTTTAATTG-3') and HpsR (5'-TTACATAGAAACTTCTTTTG-3') were synthesized by TaKaRa Biotechnology (Dalian, China) Co., Ltd. PCR amplification of the *ompA* gene of the 15 reference strains, the isolated *H. parasuis* Chinese strains and the App strains was carried out in a 25 μl reaction mix. The mix contained H_2O, 1 μl of DNA, 10 × buffer (Mg^{2+}), 2.5 μl of 2 mM dNTP, 1 μl of 10 mM HpsF and HpsR and 5 U DNA Marker Taq DNA polymerase (Takara Sake U.S.A. Inc.). The reaction conditions for the PCR were one denaturing step at 94°C for 4 min and then 35 cycles with denaturing at 94°C for 1 min, annealing 48°C for 45 sec and extension at 72°C for 80 sec. This was followed by an extension step of 72°C for 10 min. The PCR products were inspected by 2% agarose electrophoresis.

RFLP analysis: Restriction sites of the *omp*A gene were analyzed by DNAStar on *MspA1*I and *Dde*I (New England Biolabs Inc.). The 20 μl reaction for *MspA1*I contained 2 μl 10×NEB buffer, 10 μl (≤0.2 μg) PCR products, 0.2 μl 100 × BSA and 5 U *MspA1*I. Deionized water after sterilization was added to adjust the reaction mix to 20 μl, which was then incubated at 37°C for 4 hr. The reaction mix for DdeI contained 2 μl *Dde*I 10 × NEB buffer, 10 μl (≤0.2 μg) PCR products and 5 U *Dde*I. Deionized water after sterilization was added to adjust the reaction mix to 20 μl, which was then incubated at 37°C for 4 hr. Electrophoresis of the RFLP-REP products was performed on a 2% agarose gel.

RESULTS

PCR amplification of the ompA gene: The *ompA* genes of the *H. parasuis* reference strains, local isolates and App strains were amplified with the above-mentioned primers. Specific fragments of about 1104 bp were obtained by amplification of 15 *H. parasuis* reference strains and 49 local isolates, which did correspond to the size of the *ompA* gene of *H. parasuis* reported in 2009 (Fig. 1) [14]. There was no amplified band for 12 serotypes of the APP reference strains. The result of this test showed that the designed primers were specific for amplification of the *ompA* gene of *H. parasuis*.

RFLP analysis of reference strains of H. parasuis: Restriction enzyme digestion of 15 serotypes of reference strains of *H. parasuis* was carried out with the enzymes *MspA1*I and *Dde*I. Digestion of the *ompA* gene by *Dde*I and *MspA1*I resulted in 5 (Fig. 2) and 3 (Fig. 3) different RFLP patterns, respectively. Overall, 15 reference strains resulted in 8 genotypes designated as AA, BA, CA, BB, BC, BD, AE and CD (Table 1).

RFLP analysis of isolated strains of H. parasuis: The 49 isolated strains used in this test were isolated from sick or apparently healthy pigs from different pig farms in northwest China between 2008 and 2009. Genotypes AA, BA, CA, BB, BC, BD, CB and CD were identified by the RFLP analysis (Figs. 4 and 5, Table 2), and seven of these genotypes also existed in the genotypes of the reference strains. Genotype CB only existed in the isolated strains. Interestingly, genotypes BA, CD and CA were entirely from the strains isolated from diseased pigs and accounted for 38.8%, 22.4% and 18.8% of the isolates, respectively. On the other hand, the strains isolated from apparently healthy pigs were classified into genotypes AA, BB, BC and CB.

Serotype distribution: Table 3 presents the prevalence of different serotypes from the local pigs. Serotypes 5, 4 and 12 accounted for the majority (63.3%) of the strains identified. However, 22.4% of the samples did not match any of

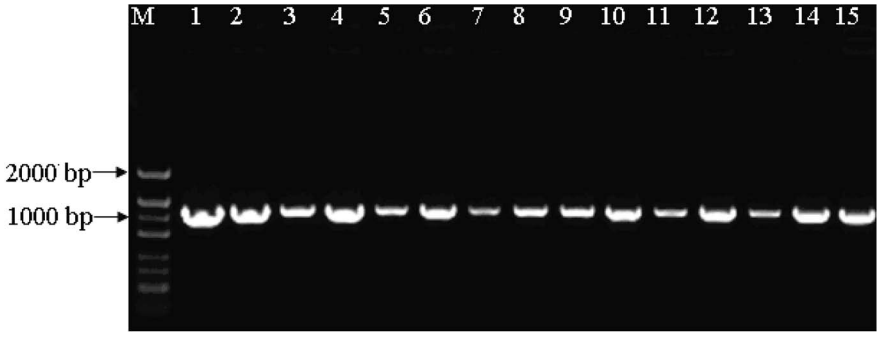

Fig. 1. PCR amplification of the *ompA* gene of Hps. M: DNA Marker. Lanes 1–15: reference Hps serotypes.

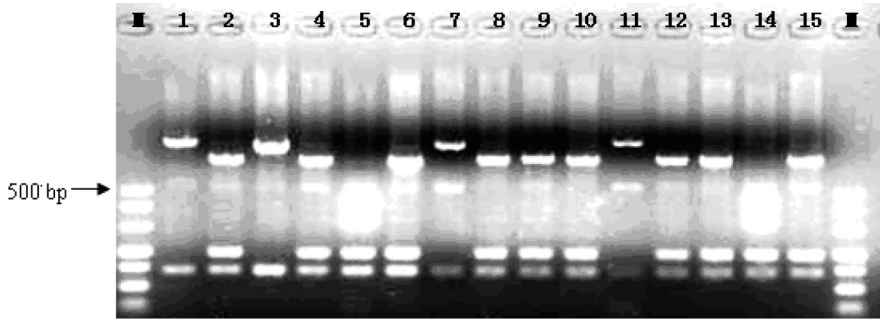

Fig. 2. Electrophoresis of the *ompA* gene of 15 Hps reference strains digested with *MspAI*I. M: DNA Marker. Lanes 1–15: reference Hps serotypes.

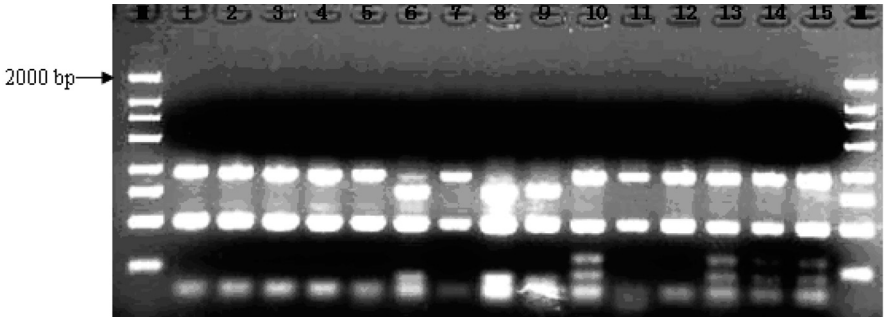

Fig. 3. Electrophoresis of the *ompA* gene of Hps reference strains digested with *Dde*I. M: DNA Marker. Lanes 1–15: reference Hps serotypes.

Table 1. Genotypes of the 15 reference samples of Hps after restriction

Reference		Restriction enzymes		Genotype
ID	Serotype (Serovar #)	MspA1I	DdeI	
NO.4	1	A	A	AA (I)
SW140	2	B	A	BA (II)
SW114	3	A	A	AA (I)
SW124	4	B	A	BA (II)
Nagasaki	5	C	A	CA (III)
131	6	B	B	BB (IV)
174	7	A	A	AA (I)
C5	8	B	B	BB (IV)
D74	9	B	C	BC (V)
H367	10	B	D	BD (VI)
H465	11	A	E	AE (VII)
H425	12	B	A	BA (II)
17975	13	B	D	BD (VI)
22113	14	C	D	CD (VIII)
15995	15	B	D	BD (VI)

the reference serotypes.

Genotype distribution: Genotype II (BA) was the most abundant genotype (38.8%) among the isolates (Table 4). Genotype III (CA) was the next most abundant (18.4%). The combination of genotypes II and III accounted for half (57.2%) of the isolated strains. Genotype VII was not observed in the isolates; however, a small portion of the isolates (4.1%) showed potentially a new genotype – CB. In addition, many serotypes were included in the same genotype (Table 4).

DISCUSSION

The outer membrane proteins (OMPs) are the main pathogenic and immune factors of *H. parasuis* [12]. This study is the 1st report to genotype the *omp*A gene by PCR-

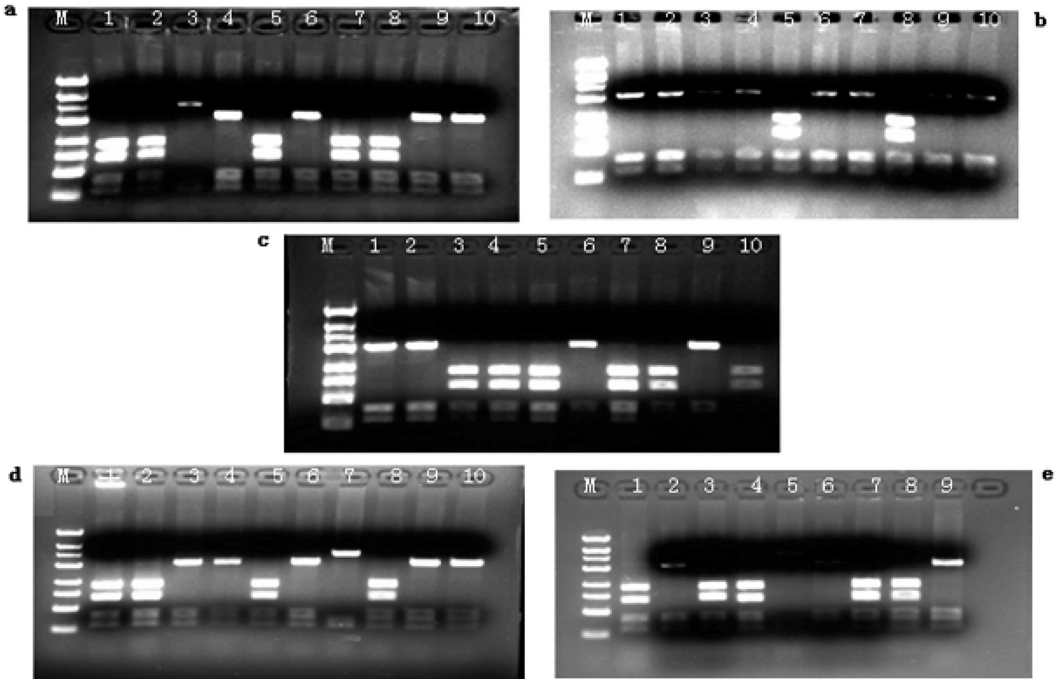

Fig. 4. Electrophoresis of the *ompA* gene of Hps isolated strains digested with *MspA*lI. M: DL2000. Panel a: Lanes 1–10 are isolated strains Hps0801- Hps0810. Panel b: Lanes 1–10 are isolated strains Hps0811-Hps0820. Panel c: Lanes 1–10 are isolated strains Hps0821-Hps0909. Panel d: Lanes 1–10 are isolated strains Hps0910-Hps0919. Panel e: Lanes 1–10 are isolated strains Hps0920- Hps0928.

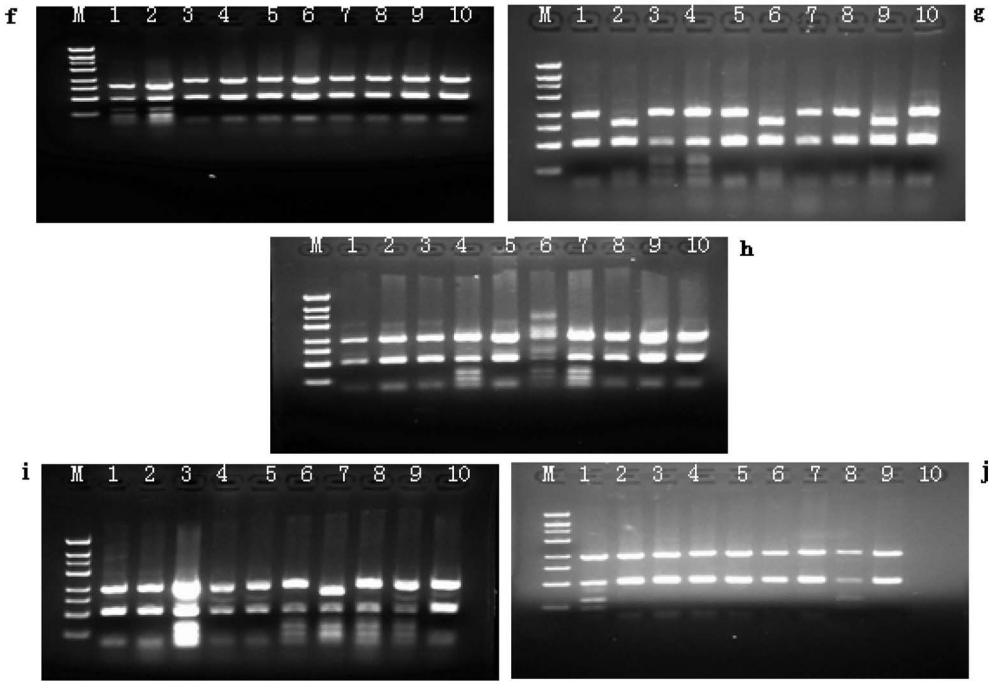

Fig. 5. Electrophoresis of the *ompA* gene of Hps isolated strains digested with *Dde*I. M: DL2000. Panel f: Lanes 1–10 are isolated strains Hps0801-Hps0810. Panel g: Lanes 1–10 are isolated strains Hps0816, Hps0820, Hps0818, Hps0815, Hps0811, Hps0814, Hps0813, Hps0817, Hps0819 and Hps0812. Panel h: Lanes 1–10 are isolated strains Hps0919, Hps0911, Hps0915, Hps0910, Hps0916, Hps0917, Hps0913, Hps0918, Hps0912 and Hps0914. Panel i: Lanes 1–10 are isolated strains Hps0921, Hps0924, Hps0920, Hps0903, Hps0927, Hps0926, Hps0922, Hps0920, Hps0923 and Hps0925. Panel j: Lanes 1–9 are isolated strains Hps0904, Hps0821, Hps0901, Hps0907, Hps0902, Hps0905, Hps0906, Hps0909 and Hps0908.

Table 2. RFLP analysis of clinical isolated strains of Hps

Sample#	Sample origin		Enzymes		Genotype	Serovar
	Tissue	Donor healthy status	MspA1I	DdeI		
Hps0801	Lung	Healthy	C	B	CB	5
Hps0802	Lung	Healthy	C	B	CB	5
Hps0803	Lung	Healthy	A	A	AA	5
Hps0804	LN (Inguinal)	Sick	B	A	BA	4
Hps0805	Joint fluid	Sick	C	A	CA	12
Hps0806	Heart + blood	Sick	B	A	BA	12
Hps0807	Lung	Sick	C	A	CA	12
Hps0808	Heart + blood	Sick	C	A	CA	12
Hps0809	Heart + blood	Sick	B	A	BA	4
Hps0810	LN[a)]	Sick	B	A	BA	N
Hps0811	Brain	Sick	B	A	BA	5
Hps0812	Heart + blood	Sick	B	A	BA	5
Hps0813	Joint fluid	Sick	B	A	BA	14
Hps0814	Nose (swab)	Healthy	B	C	BC	14
Hps0815	Heart + blood	Sick	C	D	CD	4
Hps0816	Pericardial effusion	Sick	B	A	BA	5
Hps0817	Pericardial effusion	Sick	B	A	BA	5
Hps0818	Joint fluid	Sick	C	D	CD	N
Hps0819	Nose (swab)	Healthy	B	C	BC	1
Hps0820	Nose (swab)	Healthy	B	B	BB	N
Hps0821	Joint fluid	Sick	B	A	BA	4
Hps0901	Heart + blood	Sick	B	A	BA	4
Hps0902	Endocardium	Sick	B	A	BA	12
Hps0903	Lung	Sick	C	D	CD	5
Hps0904	Heart + blood	Sick	C	D	CD	15
Hps0905	Joint fluid	Sick	B	A	BA	12
Hps0906	Plural effusion	Sick	C	A	CA	N
Hps0907	Peritoneum	Sick	C	A	CA	5
Hps0908	Brain	Sick	C	A	CA	5
Hps0909	Heart + blood	Sick	C	D	CD	14
Hps0910	Heart + blood	Sick	C	D	CD	N
Hps0911	LN	Sick	C	A	CA	14
Hps0912	Lung	Sick	B	A	BA	N
Hps0913	Brain	Sick	B	D	BD	14
Hps0914	Endocardium	Sick	C	A	CA	13
Hps0915	Peritoneum	Sick	B	A	BA	N
Hps0916	Nose (swab)	Healthy	A	A	AA	4
Hps0917	Joint fluid	Sick	C	D	CD	5
Hps0918	Blood (heart)	Sick	B	A	BA	N
Hps0919	Joint fluid	Sick	B	A	BA	5
Hps0920	Brain	Sick	C	D	CD	5
Hps0921	Brain	Sick	B	A	BA	N
Hps0922	Lung	Sick	C	D	CD	5
Hps0923	Plural effusion	Sick	C	D	CD	N
Hps0924	Lung	Healthy	A	A	AA	N
Hps0925	Joint fluid	Sick	B	A	BA	12
Hps0926	Brain	Sick	C	D	CD	4
Hps0927	Brain	Sick	C	A	CA	5
Hps0928	Nose (swab)	Healthy	B	B	BB	5

a) LN, Lymph nodes; N, unidentifiable by the reference serotypes.

RFLP in 15 reference strains and 49 isolated strains from northwest China. The result show that both the 15 reference strains and 49 field isolates are divided into 8 RFLP patterns. Among them, 7 RFLP patterns were identical between the reference and isolated strains. One new RFLP pattern, CB, was found in the isolated strains, although this genotype only accounted for 2 isolated samples.

Among the reference strains, there were 3 (serotypes 1, 3 and 7), 3 (serotypes 2, 4, and 12), 2 (serotypes 6 and 8) and 3 (serotypes 10, 13 and 15) serotypes in genotypes I (AA), II (BA), IV (BB), and VI (BD), respectively. The remaining genotypes (i.e., V, VII and VIII) had one serotype each.

Table 3. Serotype distribution of the isolated Hps strains identified by gel diffusion

GD Serotypes	No. of samples	Prevalence (%)
1	1	2.0
4	8	16.3
5	16	32.7
12	7	14.3
13	1	2.0
14	4	8.2
15	1	2.0
Unidentifiable	11	22.4
Total	49	100%

Table 4. Correlation between *ompA* genotypes and GDP serotypes in Chinese pigs

Gene Type	Abbreviation	Number identified	Prevalence (%)	Serotypes (number of isolates strains)
I	AA	3	6.1	4 (1), 5 (1), N (1)
II	BA	19	38.8	4 (4), 5 (5), 12 (4), 14 (1), N (5)
III	CA	9	18.4	5 (3), 12 (3), 13 (1), 14 (1) N (1)
IV	BB	2	4.1	5 (1), N (1)
V	BC	2	4.1	1 (1), 4 (1)
VI	BD	1	2.0	14 (1)
VII	AE	0	–	–
VIII	CD	11	22.4	4 (2), 5 (4), 14 (1), 15 (1), N (3)
	CB	2	4.1	5 (2)

Specifically, serotypes 9, 11 and 14 reflected genotypes V, VII and VIII, respectively.

Each of the 15 reference strains of *H. parasuis* were incubated via the abdominal cavity in SPF pigs by Kielstein and Rapp-Gabrielson, and the serotypes were divided into three groups (high, moderate and non-virulent strain) according to their virulence judged by the incidence, mortality and clinical symptoms in affected pigs. Among them, highly virulent strains included serovars 1, 5, 10, 12, 13 and 14; moderately or mildly virulent strains included serovars 2, 4, 8 and 15; and non-virulent strains included serovars 3, 6, 7, 9 and 11 [6, 9]. Six genotypes determined by the RFLP-PCR method are classified into virulent and non-virulent strains, except for genotypes I and IV, according to the results of classification of 15 reference serovars of *H. parasuis* by Kielstein *et al.* [6]. Among them, the virulent strains include genotypes II (BA), III (CA), VI (BD) and VIII (CD); the non-virulent strains include genotypes V (BC) and VII (AE).

In the present study, PCR-RFLP analysis of 49 isolated strains from pig farms in northwest China showed that the most prevalent genotypes were II (BA), III (CA) and VIII (CD). Interestingly, all strains isolated from diseased pigs were distributed in four genotypes, II (BA), III (CA), VIII (CD) and VI (BD). On the other hand, most strains isolated from apparently healthy pigs were classified into genotypes AA, BB, BC and CB. However, the virulent reference serovar 1 strain has an AA genotype, and the fact that nearly all strains from the healthy pigs belong to serovars classed as virulent suggests that these genotypes might also include virulent strains. Further validation with more field strains is needed. Only genotype CB had a different genotype pattern than the reference strains of *H. parasuis* in this study.

Serotyping by heat-stable antigens of isolated strains of *H. parasuis* extracted in this study by gel diffusion showed that the majority of *H. parasuis* strains in northwest China belong to serotypes 4, 5, 12 and 14. About 22.4% of the isolated strains could not be typed by this method in this study, which basically coincides with the results of Cai *et al.* [2]. The results also showed that the isolated strains with the same serotype may not have the same RFLP pattern; the same RFLP pattern contained different serotypes, which indicated no necessary correlation between genotype and serotype.

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