Development of Serotype-Specific PCR Assays for Typing of *Haemophilus parasuis* Isolates Circulating in Southern China

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ABSTRACT The bacterium *Haemophilus parasuis* is the specific pathogenic cause of Glässer's disease in swine. Fifteen serotypes of *H. parasuis* have been reported. A method to serotype *H. parasuis* isolates accurately would help to prevent and control Glässer's disease outbreaks through appropriate vaccination and to understand the epidemiology in specific geographic areas. However, according to traditional serotyping, the rate of nontypeable (NT) strains is 10 to 40%, which gives low accuracy. In the present study, we developed a set of PCR assays that are able to identify all the currently known *H. parasuis* serotypes, with a detection limit of 5 CFU. This PCR method is particularly useful to distinguish serotype 5 from serotype 12. We then surveyed the serotype prevalence of *H. parasuis* isolates from southern China using both the traditional indirect hemagglutination (IHA) and current PCR methods. Of the 298 isolates tested, 228 (76.51%) and 281 (94.30%) were serotyped by the IHA and PCR tests, respectively, with a concordance rate of 80.87% (241/298). The most prevalent serotypes obtained by PCR were 4, 5, 12, 13, NT, and 2, and the most prevalent obtained by IHA were NT, 5, 4, 12, 13, and 2. In conclusion, the PCR assays developed in this study provide a rapid and specific method for the molecular serotyping of *H. parasuis*.

KEYWORDS serotyping, serotype-specific PCR, indirect hemagglutination assay, *Haemophilus parasuis*

The Gram-negative bacterium *Haemophilus parasuis* is the etiological pathogen of Glässer's disease, which results in polyserositis syndrome with pleuritis, peritonitis, meningitis, and arthritis in swine (1, 2) and causes serious economic losses in the swine industry. *H. parasuis* is an opportunistic pathogen (3) that often coinfects with other swine pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (4–7). At present, 15 serotypes of *H. parasuis* have been identified (7, 8). To improve understanding of the molecular epidemiological characteristics of *H. parasuis* infection in specific geographic regions and aid clinical prevention and medication, it is very important to identify the serotypes of isolated strains rapidly.

Much research has been conducted regarding the worldwide distribution of *H. parasuis* serotypes. For example, serotypes 5, 4, 13, and 2 are dominant in some European countries (9–11), while in Australia, serotypes 4, 5, and 13 are the most prevalent (12). In Brazil, the prevalent serotypes are 4, 5, 14, and 13 (13), and in North America, serotypes 4, 5, 13, and 7 are prevalent (14). In addition, epidemics of *H. parasuis* infection have shown different characteristics at various times. Serotypes 4, 5,

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FIG 1 Band patterns of serotyping PCR for all 15 serovars of *H. parasuis* reference strains. Lanes S1 to S15, 15 serovars of *H. parasuis* reference strains; lane M, DL2000 DNA molecular weight marker; H₂O, negative control.

13, 14, and 12 and nontypeable (NT) strains were the most prevalent in 17 provinces of China during the year 2005 (15) and in southern China during 2011 (16), but during 2016 the serotypes 5, 4, NT, 7, and 13 were predominant in 7 provinces of China (17). Yet, the global epidemiology of *H. parasuis* serotypes remains unclear.

The accuracy of the traditional serotyping methods is limited and often results in 10% to 40% rates of NT serotypes (9, 10, 12, 14–16, 18–21). Currently, the conventional tests for identification of *H. parasuis* serotypes using serotype-specific antisera are gel immunodiffusion and indirect hemagglutination assay (IHA) (19, 20). These methods require serotype-specific antisera (22–25). However, it is time-consuming to produce these antisera, and the detection methods are often costly (12, 19, 20, 26–28). The IHA method is more useful than the gel immunodiffusion method, being able to detect 15% more typeable serotypes (15, 29). PCR-based serotyping using primers that can be used to amplify serotype-specific genes is the most effective and rapid method for the identification and differentiation of serotypes. PCR assays used to identify specific *H. parasuis* serotypes have been reported (17, 29).

Historically, the *H. parasuis* serovar was believed to produce heat-stable antigens, such as capsular polysaccharide (CPS) antigen and lipopolysaccharide (LPS) or lipooligosaccharide (LOS), in a strain-dependent manner (11). There are many genes located at the CPS locus (11, 17, 29). Although it is simple and rapid to identify *H. parasuis* serotypes using PCR amplification of the serotype-specific CPS gene, serotypes 5 and 12 cannot be differentiated using the currently available PCR assays (17, 29). Therefore, an improved rapid and accurate PCR method with a high detection rate is required.

In the present study, we developed a rapid PCR serotyping method for detection of *H. parasuis* reference strains and clinical samples, which is especially useful to differentiate serotype 5 from serotype 12. The results of our method were then compared and confirmed with reference to IHAs.

RESULTS

Development of the PCR typing methods. In the present study, the 15 serotype-specific PCR amplification products were electrophoresed (Fig. 1) and confirmed by DNA sequencing. Specific amplification bands were obtained using the primers. However, the PCR assay targeted for amplifying the *funK* gene could not differentiate serotype 5 from serotype 12.

A comparative analysis of the genome sequences of serotypes 5 and 12 ([CP001321.1](#) and [CP005384.1](#)) was performed using Mauve 2.3.1 software, and specific primer H12

TABLE 1 Distribution of *H. parasuis* isolates in organs of pigs with Glässer's disease from 2007 to 2015

Yr	No. of isolates by organ:									Total
	Lung	Brain	Joint	Heart	Liver	Tonsil	Spleen	Lymph	Unknown	
2007	7	1	2	1				1		12
2008	22	1	9		7			3	5	47
2009	20		4	3					5	32
2010	10	1								11
2011	32	8	2	1	2	6	1		1	53
2012	48	2	1	7		13			4	75
2013	9		1	1		9			1	21
2014	11			2	1	7				21
2015	20	1	1						4	26
Total	179	14	20	15	10	35	1	4	20	298

(used to amplify a hypothetical gene) was designed to differentiate the serotype 5 and serotype 12 strains. The PCR assay using primer H12 resulted in a specific positive band for serotype 12 but not for serotype 5 (Fig. 1). As few as 5 CFU ($0.5 \mu\text{l}$, 1×10^4 CFU/ml) of each reference strain, 15 serotypes in broth culture, could be detected in one PCR (25 μl). The specificity of the method was good, and there was no cross-reaction among the 15 serotypes. There was also no reactivity against closely related commensal *Pasteurellaceae* and other bacterial pathogens of pigs.

Comparison of the prevalence and serotype profiles between PCR and IHA. A total of 298 Chinese *H. parasuis* clinical isolates were used for evaluation of our PCR method. Among them, 179 were isolated from lung, 14 from brain, 20 from joint fluid, 15 from heart, 10 from liver, 35 from tonsil, 1 from spleen, 4 from lymph, and 20 from unknown isolation sites (Table 1). These *H. parasuis* isolates from pigs with clinical signs of Glässer's disease were examined using the PCR and IHA methods. Of the 298 isolates, 17 (5.7%) were confirmed as nontypeable by PCR (Table 2 and Fig. 2). In contrast, 70 isolates were confirmed as nontypeable strains by IHA. The most prevalent serotype identified by PCR was serotype 4 (25.17% of isolates) and then serotypes 5 (23.15%), 12 (20.47%), 13 (6.04%), NT (5.70%), 2 (5.03%), 7 (3.69%), 1 (3.36%), 10 (2.01%), 14 (2.01%), 11 (1.34%), 8 (1.00%), 9 (0.67%), and 6 (0.34%) (Fig. 3). The most prevalent serotype profile identified by IHA was NT (23.49%) and then serotypes 5 (18.46%), 4 (18.12%), 12 (16.11%), 13 (5.03%), 2 (5.03%), 7 (3.02%), 1 (2.68%), 14 (2.01%), 10 (1.68%), 11 (1.34%), 15 (1.34%), 8 (1.00%), and 9 (0.67%) (Table 2 and Fig. 2).

TABLE 2 Summary of the PCR and IHA results from 298 *H. parasuis* isolates

Serotype determined by PCR	No. of isolates with serotype determined by IHA															NT ^a	Total
	1	2	4	5	6	7	8	9	10	11	12	13	14	15			
1	8															2	10
2		15															15
4			54													21	75
5				55												14	69
6																1	1
7						9										2	11
8							3										3
9								2									2
10									5							1	6
11										4							4
12											48					13	61
13												15				3	18
14													6				6
15																	0
NT ^a															4	13	17
Total	8	15	54	55	0	9	3	2	5	4	48	15	6	4	70		298

^aNT, nontypeable isolates.

FIG 2 Serotype distribution of 298 Chinese isolates as determined by IHA and PCR.

Of the 228 serotyped isolates from the IHA, 61 were discrepant with PCR results. These serotype-discrepant isolates included a serotype 15 isolate identified as NT by PCR, 2 NT isolates identified as serotype 1, 21 NT isolates identified as serotype 4, 14 NT isolates identified as serotype 5, 1 NT isolate identified as serotype 6, 2 NT isolates identified as serotype 7, 1 NT isolate identified as serotype 10, 13 NT isolates identified as serotype 12, and 3 NT isolates identified as serotype 13 (Table 2). For the 228 typeable isolates identified by IHA, the concordance with PCR was >98.25% (224/228).

Distribution of serotypes by PCR of *H. parasuis* isolated from diseased pigs in southern China between 2007 and 2015. The serotypes of 298 clinical *H. parasuis* pathogens isolated from 2007 to 2015 were identified by PCR (Table 3). Serotypes 4, 5, and 12 were the most prevalent serotypes throughout the 9 years. These 3 serotypes of *H. parasuis* were isolated nearly every year; serotype 4 was not isolated in 2010. Serotype 13 was also frequently isolated; it was isolated every year except 2007 and 2014. However, it is worth mentioning that the amount of detectable serotype 13, as a highly virulent serotype, was smaller than 4 isolates each year. Serotypes 6, 8, and 9 were the least often isolated serotypes among all the 15 serotypes.

DISCUSSION

In this study, we developed a rapid PCR typing method using different primer sequences. This method was used to identify all 15 serotypes of *H. parasuis* reference strains and 298 serotypes of clinical isolates. The PCR serotyping experiment was compared and confirmed by IHAs. Serotypes 4, 5, and 12 were identified as the most prevalent serotypes among the 298 clinical isolates.

Serotypes of *H. parasuis* are determined by antigenic regions of capsular polysaccharides, which are composed of diversified monosaccharide units. These monosaccharide units contribute to the variation of different serotypes (28, 30). The genes expressing capsular polysaccharides are clustered in the CPS locus. Until now, no

FIG 3 Serotype distribution of *H. parasuis* isolated from diseased pigs in southern China between 2007 and 2015.

TABLE 3 Summary of the PCR serotyping results of *H. parasuis* isolated from diseased pigs in 6 provinces of southern China between 2007 and 2015

Serotype	No. of isolates by yr of isolation								
	2007	2008	2009	2010	2011	2012	2013	2014	2015
1		2				2	2	2	2
2		1	4		3	2	2	3	
4	6	11	2		21	25	5	3	2
5	3	17	10	1	11	12	2	10	3
6				1					
7				1	6	2			2
8			2	1					
9									2
10					1	4		1	
11		1				2	1		
12	3	9	5	4	5	22	7	2	4
13		2	3	1	4	3	2		3
14			2						4
NT ^a		4	4	2	2	1			4

^aNT, nontypeable isolates.

available genes could be used to distinguish serotype 5 from serotype 12, due to the similar structure of the CPS gene clusters. Howell et al. (29) developed multiplex PCR (mPCR) assays for serotypes 1 to 15 but not 5 and 12. The mPCR results were in 90% concordance with the IHA serotyping results, enabling the differentiation of 14 of the 15 serovars of *H. parasuis*. In a recent report by Ma et al. (17), serotypes 5 and 12 also could not be distinguished. In their research, 73 (73%) and 93 (93%) were serotyped by the gel immunodiffusion test and mPCR, respectively, with a concordance rate of 66% (66/100). Therefore, in the present study the choice of gene targets for serotype specificity was an important consideration in developing the PCR serotyping assays. We designed a new primer, H12, that can be used for detection of serotype 12. This PCR method could detect more typeable serotypes than the IHA, with greater than 98.25% (224/228) concordance with the IHA, and was particularly useful to distinguish serotype 5 from serotype 12. The method is rapid and effective for identification of all the *H. parasuis* serotypes.

Serotyping *H. parasuis* helps us to understand the epidemiology of disease and to monitor the serotype prevalence, as well as to provide information for vaccine development. The highly virulent serotypes of *H. parasuis* are 1, 5, 10, 12, 13, and 14 and some clade 4 serovars. Serotypes 2, 8, and 15 are less virulent, and the avirulent serotypes are 3, 6, 7, 9, and 11 (4, 19, 31–33). In the present study, serovars 4 (25.16%), 5 (23.15%), and 12 (20.47%) were the most prevalent, which is consistent with other findings (15–17). However, Zhang et al. (16) reported that serotypes 5, 4, and 2 were the most predominant. In the work of Zhang et al., 112 *H. parasuis* strains were subjected to serovar analysis by gel diffusion tests and IHAs. The difference in prevalence of *H. parasuis* serotypes may be due to lower sample volume, different sampling locations, or different serotyping methods. Serotype 3 of *H. parasuis* was not isolated in the present study, which is consistent with the reports of Ma et al. (17) and Cai et al. (15). It was also reported that only 1 strain with serotype 3 was isolated in southern China, indicating that serotype 3 is rare (16). Three strains of serotype 8 were isolated in the present study. Serotype 8 of *H. parasuis* was also reported by Zhang et al. (16) but not reported by Ma et al. (17) or Cai et al. (15), indicating that an epidemic of serotype 8 of *H. parasuis* may be related to regional characteristics.

In our study, 17 of the 298 isolates (5.7%) were confirmed by PCR as nontypeable. The ratio of NT to total was close to that reported by Ma et al. (17). In the study by Ma et al., 27% and 7% of NT isolates were serotyped using the gel immunodiffusion test and the multiplex PCR method, respectively. The NT isolates have been reported to contain obvious deletions and/or unknown sequences in the serotype-specific region of their capsule loci with “No Significantly Similar Sequence” (NSSS) found via BLASTn

TABLE 4 Serovar distribution of *H. parasuis* isolates in organs of pigs with Glässer's disease

Serotype	No. of isolates by organ:									Total
	Lung	Brain	Joint	Heart	Liver	Tonsil	Spleen	Lymph	Unknown	
1	7		2			1				10
2	7	2				5	1			15
3										
4	45	3	6	3	3	9		3	3	75
5	35	6	3	6	4	6		1	8	69
6	1									1
7	8					2			1	11
8	3									3
9	2									2
10	1					3			2	6
11	1					2			1	4
12	37	2	6	5	3	5			3	61
13	12	1	1	1		2			1	18
14	5								1	6
15										0
NT	15		2							17
Total	179	14	20	15	10	35	1	4	20	298

search. This may be the reason why there are such a high number of isolates that are not typeable by PCR. In the present study, serotypes 4, 5, and 12 were detected as the most prevalent over the 9 years; these serotypes were isolated every year from 2007 to 2015. It is worthwhile to note that a large number of highly virulent serotype 13 or moderate virulent serotype 2 (4, 19, 31–33) strain isolates were also collected, which can provide scientific evidence for work toward prevention.

In this study, *H. parasuis* isolates were collected from multiple tissues of pigs with classical signs of Glässer's disease, including lung, brain, joint, heart, liver, tonsil, spleen, and lymph. Among them, the greatest number of isolates was found in the lung, followed by the tonsils, which may be because *H. parasuis* is a species of commensal bacteria in the swine upper respiratory tract. The results allow us to isolate *H. parasuis* easily from lung tissue. The distribution of different serotypes was not correlated with specific organs, and there were multiple serotypes isolated from different organs (Table 4). All serotypes were isolated in the lungs, except for types 3 and 15. Serotype 4, 5, 12, and 13 strains were isolated from lung, brain, joint, heart, liver, or tonsil. In addition, it was noted that *H. parasuis* was often isolated together with *Streptococcus suis*. Therefore, it is well to consider the prevention and treatment of mixed infections of *H. parasuis* and *S. suis* on pig farms.

In conclusion, the PCR assays developed in this study provide a rapid and specific

TABLE 5 Characteristics of 15 serovars of *H. parasuis* reference strains

Serovar	Reference strain	Isolation site	Diagnosis	Country of origin
1	No. 4	Nose	Healthy	Japan
2	SW140	Nose	Healthy	Japan
3	SW114	Nose	Healthy	Japan
4	SW124	Nose	Healthy	Japan
5	Nagasaki	Meninges	Septicemia	Japan
6	131	Nose	Healthy	Switzerland
7	174	Nose	Healthy	Switzerland
8	C5	Unknown	Unknown	Sweden
9	D74	Unknown	Unknown	Sweden
10	H367	Nose	Healthy	Germany
11	H465	Trachea	Pneumonia	Germany
12	H425	Lung	Polyserositis	Germany
13	IA-84-17975	Lung	Unknown	USA
14	IA-84-22113	Joint	Unknown	USA
15	SD-84-15995	Lung	Pneumonia	USA

TABLE 6 Primers of 15 genes used for PCR amplification of 15 serotypes of *H. parasuis*

Serotype	Direction	Howell et al. (29)		Current study					
		Targeted gene	Sequence (5' to 3')	Product size, bp	Targeted gene	Sequence (5' to 3')	Product size, bp	Temp, °C	Location of primer (positions)
1	Forward Reverse	<i>funB</i>	CTGTGTATATCTACCCCGATCATCAGC GTCCAACAGAAATTTGGACCAATTCCTG	180	<i>funB</i>	TGCATAAAAAATTTTGGAA TTATATATATTTTACATTTCTAAG	1,245	49	1–1245
2	Forward Reverse	<i>wzx</i>	CTAACAGTTAGGTATGGAGGGTTTGGTG GGCAGCTGAATTAAGGATTAATTGACTG	295	<i>funE</i>	ATGGAAGAAAAAGAATATATC TTAAAGTTTGTATTGTCAATG	1,032	52	1–1032
3	Forward Reverse	<i>glyC</i>	CATGGTTTATCTGACTTGGCTGT TCCACATGAGGCCGTTCTAATATACT	650	<i>dgdA</i>	ATGACTAAAAAAATTTAGTTTACAG TTACTTAATACCTAAGCG	1,068	52	1–1068
4	Forward Reverse	<i>wcIP</i>	GGTTAAGAGTAGAGCTAAGAATAGAGG CTTTCACAAACAGCTTAGAAACC	320	<i>gltG</i>	ATGAATATAAAGTCTCAATTATTA TTACATATGTTTACAATTCC	753	52	1–753
5 or 12	Forward Reverse	<i>wcWk</i>	CCACTGGATAGAGAGTGGCAGG CCATACATCTGAATTTCCTAAGC	450	<i>funk</i>	ATGCCAATAGAGATAGC CCTGCCATATTATGA	560	52	1–560
	Forward Reverse	<i>gltI</i>	GATTCTGATGATTTTGGCTGACGGAACG CCTATTCTGTCTATAAGCATAGACAGG	360	<i>funL</i>	ATGAGTATTTTTTCTAATTG TTCCCTGATCATTTGTAGTAACC	443	52	1–443
7	Forward Reverse	<i>funQ</i>	CTCCGATTTCATCTTTTCTATGTGG CGATAAACCATTAACAATTCCTGGCAC	490	<i>funQ</i>	TAGTTGGTATATTAATTTCT AGAATGCATCTGTACCACATAAG	600	52	239–838
8	Forward Reverse	<i>scdA</i>	GGAAGGGGATTACTACTACTGAAAG CTCCATAGAACTGCTGCTTGAG	650	<i>scdA</i>	CAGCAGGTTCTATGAGTCA CACATTATAACTTTCTTT	350	49	851–1200
9	Forward Reverse	<i>funV</i>	AGCCACATCAATTTTAGCCTCATCA CCTTAATAGCCTATGTCTGTACC	710	<i>funV</i>	GCTCCAATATCAGACAGTA AGAGTAATGAGCATCTCCG	819	58	82–900
10	Forward Reverse	<i>funX</i>	GGTGACATTTATGGCGAGTAAGTC GCACGTGCATCAATTAACAATCTTAAAGACG	790	<i>funX</i>	TGATTATTTACTGCGCTTTTA CACCTAGCGTAAACCATATA	320	55	566–886
11	Forward Reverse	<i>amIA</i>	CCATCTCTTTAACTAATGGAGCTG GGACGCCAACCAAGTATTATCAAAATG	890	<i>actA</i>	ATGATTATAGTATTATATGTC CTATTATTTTTTGAATAATTCTC	657	52	1–657
12	Forward Reverse				Hypothetical gene	ATGGCTCAGATCCGAAAG ATTTCCTTTCTTAAACGC	508	60	1–508
13	Forward Reverse	<i>gltP</i>	GCTGAGAGAGTTGAAGAAGTTGTAC CAATCAAAATGAAACAACAGGAAGC	840	<i>walL</i>	GGCATTAGAGTTTCACTTA TATTAGCATACCCAGCAT	800	60	102–901
14	Forward Reverse	<i>funAB</i>	GCTGGTTATGACTATTTCTTTCGGG GCTCCCAAGATTAAACCACAAAGCAAG	730	<i>funAB</i>	TGCTTTGTACTACTAATTATTG TAGTAACCTCCAGATAAAGC	906	51	5–910
15	Forward Reverse	<i>funI</i>	CAAGTTGGATTGGAGCATATATC CCTATATCATTTTGTGGATGTACG	550	<i>funI</i>	TTGGAAGTATAAGGAGCT GATGTAGCCATTAAGTCAAT	536	62	109–644

method for the molecular serotyping of *H. parasuis*. This PCR method is particularly useful to distinguish serotype 5 from serotype 12. Serotypes 4, 5, and 12 were identified as the most prevalent serotypes among the 298 clinical isolates.

MATERIALS AND METHODS

Ethics statement. The Animal Ethics Committee of South China Agricultural University approved this study. The porcine reproductive and respiratory syndrome virus (PRRSV)-negative nursery pigs used in this study were treated in strict accordance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China. All animals were euthanized humanely using sodium pentobarbital anesthesia to reduce suffering.

Bacterial strains. Reference strains of *H. parasuis*, comprising serotypes 1 to 15, were provided by South China Agricultural University in Guangdong province (Table 5). A total of 298 *H. parasuis* clinical strains, isolated between 2007 and 2015 from 894 pigs with Glässer's disease in 113 farms in Guangdong, Jiangxi, Guangxi, Sichuan, Hunan, and Henan provinces of southern China, were used for evaluation of a PCR method of *H. parasuis* serotyping and for understanding the molecular epidemiological characteristics of *H. parasuis*. The isolates were all characterized as *H. parasuis* by detecting their biochemical characteristics (NAD dependent), Gram staining properties, and 16S rRNA sequences (99 to 100% identical to *H. parasuis* strain H0165 [NCBI accession no. ABKM01000007]).

PCR typing methods. By comparing the genomic differences of 15 serotypes of *H. parasuis*, primers for each serotype-specific gene located in the capsular polysaccharide synthesis gene clusters were designed. The type-specific primer sequences, annealing temperatures, and PCR amplification product sizes of 15 target genes used for identification of 15 serotypes of *H. parasuis* reference strains are summarized in Table 2. The specificity of 15 pairs of primers was verified by PCR, and they were then used to identify each specific serotype. Of note, the primer sequences of the genes *funB*, *funQ*, *scdA*, *funV*, *funX*, and *funAB* are different from those reported (Table 6) by Howell et al. (11), although the primer names are the same. In addition, the primer sequences of the genes *funE*, *dgdA*, *gltG*, *funL*, *actA*, *waal*, and *funJ* used for identification of serotypes 2, 3, 4, 6, 11, 13, and 15, respectively, were also different from recent reports by Howell et al. (11).

The serotypes of the 15 *H. parasuis* reference strains and the 298 clinical isolates were detected by PCR typing methods. In brief, the 25- μ l PCR mixture consisted of the following: 1.25 U *Taq* DNA polymerase (TaKaRa, Japan), 2.5 μ l of 10 $\times$ PCR buffer, 2 μ l deoxynucleoside triphosphate (dNTP) mixture (0.25 mM for each dNTP), 0.5 μ l of forward and reverse primers (50 μ M), and 0.5 μ l bacterial culture. The genomic DNA was first denatured at 94°C for 5 min and then by 30 cycles of 94°C for 30 s, the annealing temperature of each serotype for 30 s, 72°C for 45 s, and a final extension at 72°C for 5 min. The PCR amplification products of the 15 *H. parasuis* reference strains were then electrophoresed in 1.0% agarose gels. The gels were observed using a gel imaging system (Tanon 4120; Tanon Science and Technology, Shanghai, China). Tris-borate buffer and a DL2000 DNA marker (TaKaRa, Japan) as the molecular weight standard were used for this gel electrophoresis. The PCR experiments were repeated twice for each bacterial strain.

To evaluate the sensitivity of the PCR assays, the *H. parasuis* reference strains were diluted in a 10-fold series, from 1×10^7 to 1×10^0 CFU/ml, and detected by the PCR typing methods. The methods were also used to detect 298 *H. parasuis* isolates from pigs in southern China, from broth cultures.

IHA. All of the 298 clinical isolates were subjected to serotyping analysis by IHA (34). The antiserum used for serotyping was produced by rabbits as previously described (14). Bacterial cells were grown overnight on tryptic soy agar (BD Company, USA) supplemented with 10% serum and 10 μ g/ml NAD. The saline extracts used as antigens were as described by Del Rio et al. (26, 35). The serotyping procedure was performed as described previously (20, 26, 35).

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All authors declare that there is no conflict of interest involved.

REFERENCES

- Costa-Hurtado M, Aragon V. 2013. Advances in the quest for virulence factors of *Haemophilus parasuis*. *Vet J* 198:571–576. <https://doi.org/10.1016/j.tvjl.2013.08.027>.
- Aragon V, Segalés J, Oliveira S. 2012. Glässer's disease, p 760–769. In Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (ed), *Diseases of swine*. Wiley-Blackwell, Chichester, United Kingdom.
- Zhang B, Tang C, Liao M, Yue H. 2014. Update on the pathogenesis of *Haemophilus parasuis* infection and virulence factors. *Vet Microbiol* 168: 1–7. <https://doi.org/10.1016/j.vetmic.2013.07.027>.
- Oliveira S, Pijoan C. 2004. *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Vet Microbiol* 99:1–12. <https://doi.org/10.1016/j.vetmic.2003.12.001>.
- Palzer A, Haedke K, Heinritzi K, Zoels S, Ladinig A, Ritzmann M. 2015. Associations among *Haemophilus parasuis*, *Mycoplasma hyorhinis*, and

- porcine reproductive and respiratory syndrome virus infections in pigs with polyserositis. *Can Vet J* 56:285–287.
6. Yu J, Wu J, Zhang Y, Guo L, Cong X, Du Y, Li J, Sun W, Shi J, Peng J, Yin F, Wang D, Zhao P, Wang J. 2012. Concurrent highly pathogenic porcine reproductive and respiratory syndrome virus infection accelerates *Haemophilus parasuis* infection in conventional pigs. *Vet Microbiol* 158: 316–321. <https://doi.org/10.1016/j.vetmic.2012.03.001>.
 7. Howell KJ, Weinert LA, Chaudhuri RR, Luan SL, Peters SE, Corander J, Harris D, Angen O, Aragon V, Bensaid A, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Holden MT, Tucker AW, Maskell DJ, BRADP1T Consortium. 2014. The use of genome wide association methods to investigate pathogenicity, population structure and serovar in *Haemophilus parasuis*. *BMC Genomics* 15:1179. <https://doi.org/10.1186/1471-2164-15-1179>.
 8. McCaig WD, Loving CL, Hughes HR, Brockmeier SL. 2016. Characterization and vaccine potential of outer membrane vesicles produced by *Haemophilus parasuis*. *PLoS One* 11:e0149132. <https://doi.org/10.1371/journal.pone.0149132>.
 9. Rubies X, Kielstein P, Costa L, Riera P, Artigas C, Espuna E. 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. *Vet Microbiol* 66:245–248. [https://doi.org/10.1016/S0378-1135\(99\)00007-3](https://doi.org/10.1016/S0378-1135(99)00007-3).
 10. Angen O, Svensmark B, Mittal KR. 2004. Serological characterization of Danish *Haemophilus parasuis* isolates. *Vet Microbiol* 103:255–258. <https://doi.org/10.1016/j.vetmic.2004.07.013>.
 11. Howell KJ, Weinert LA, Luan SL, Peters SE, Chaudhuri RR, Harris D, Angen O, Aragon V, Parkhill J, Langford PR, Rycroft AN, Wren BW, Tucker AW, Maskell DJ, BRADP1T Consortium. 2013. Gene content and diversity of the loci encoding biosynthesis of capsular polysaccharides of the 15 serovar reference strains of *Haemophilus parasuis*. *J Bacteriol* 195: 4264–4273. <https://doi.org/10.1128/JB.00471-13>.
 12. Turni C, Blackall PJ. 2010. Serovar profiling of *Haemophilus parasuis* on Australian farms by sampling live pigs. *Aust Vet J* 88:255–259. <https://doi.org/10.1111/j.1751-0813.2010.00592.x>.
 13. Castilla KS, de Gobbi DD, Moreno LZ, Paixao R, Coutinho TA, dos Santos JL, Moreno AM. 2012. Characterization of *Haemophilus parasuis* isolated from Brazilian swine through serotyping, AFLP and PFGE. *Res Vet Sci* 92:366–371. <https://doi.org/10.1016/j.rvsc.2011.04.006>.
 14. Rapp-Gabrielson VJ, Gabrielson DA. 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. *Am J Vet Res* 53:659–664.
 15. Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, Jin M. 2005. Serological characterization of *Haemophilus parasuis* isolates from China. *Vet Microbiol* 111:231–236. <https://doi.org/10.1016/j.vetmic.2005.07.007>.
 16. Zhang J, Xu C, Guo L, Shen H, Deng X, Ke C, Ke B, Zhang B, Li A, Ren T, Liao M. 2012. Prevalence and characterization of genotypic diversity of *Haemophilus parasuis* isolates from southern China. *Can J Vet Res* 76: 224–229.
 17. Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J, Liu Y. 2016. Characterization of Chinese *Haemophilus parasuis* isolates by traditional serotyping and molecular serotyping methods. *PLoS One* 11: e0168903. <https://doi.org/10.1371/journal.pone.0168903>.
 18. Tadjine M, Mittal KR, Bourdon S, Gottschalk M. 2004. Development of a new serological test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence in North America. *J Clin Microbiol* 42:839–840. <https://doi.org/10.1128/JCM.42.2.839-840.2004>.
 19. Kielstein P, Rapp-Gabrielson VJ. 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *J Clin Microbiol* 30:862–865.
 20. Rafiee M, Blackall PJ. 2000. Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for *Haemophilus parasuis*. *Aust Vet J* 78:172–174. <https://doi.org/10.1111/j.1751-0813.2000.tb10586.x>.
 21. Oliveira S, Blackall PJ, Pijoan C. 2003. Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. *Am J Vet Res* 64:435–442. <https://doi.org/10.2460/ajvr.2003.64.435>.
 22. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kalltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 *pneumococcal* serotypes. *PLoS Genet* 2:e31. <https://doi.org/10.1371/journal.pgen.0020031>.
 23. Boyce JD, Chung JY, Adler B. 2000. Genetic organisation of the capsule biosynthetic locus of *Pasteurella multocida* M1404 (B:2). *Vet Microbiol* 72:121–134. [https://doi.org/10.1016/S0378-1135\(99\)00193-5](https://doi.org/10.1016/S0378-1135(99)00193-5).
 24. Verdier I, Durand G, Bes M, Taylor KL, Lina G, Vandenesch F, Fattom AI, Etienne J. 2007. Identification of the capsular polysaccharides in *Staphylococcus aureus* clinical isolates by PCR and agglutination tests. *J Clin Microbiol* 45:725–729. <https://doi.org/10.1128/JCM.01572-06>.
 25. Onokodi JK, Wauters G. 1981. Capsular typing of *klebsiellae* by coagglutination and latex agglutination. *J Clin Microbiol* 13:609–612.
 26. Del Rio ML, Gutierrez CB, Rodriguez Ferri EF. 2003. Value of indirect hemagglutination and coagglutination tests for serotyping *Haemophilus parasuis*. *J Clin Microbiol* 41:880–882. <https://doi.org/10.1128/JCM.41.2.880-882.2003>.
 27. Morozumi T, Nicolet J. 1986. Morphological variations of *Haemophilus parasuis* strains. *J Clin Microbiol* 23:138–142.
 28. Wang K, Sun X, Lu C. 2012. Development of rapid serotype-specific PCR assays for eight serotypes of *Streptococcus suis*. *J Clin Microbiol* 50: 3329–3334. <https://doi.org/10.1128/JCM.01584-12>.
 29. Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen O, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW, BRADP1T Consortium. 2015. Development of a multiplex PCR assay for rapid molecular serotyping of *Haemophilus parasuis*. *J Clin Microbiol* 53:3812–3821. <https://doi.org/10.1128/JCM.01991-15>.
 30. Roberts IS. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu Rev Microbiol* 50:285–315. <https://doi.org/10.1146/annurev.micro.50.1.285>.
 31. Salter SJ, Hinds J, Gould KA, Lamberts L, Hanage WP, Antonio M, Turner P, Hermans PW, Bootsma HJ, O'Brien KL, Bentley SD. 2012. Variation at the capsule locus, cps, of mistyped and non-typable *Streptococcus pneumoniae* isolates. *Microbiology* 158:1560–1569. <https://doi.org/10.1099/mic.0.056580-0>.
 32. Zhang Y, Li G, Xie F, Liu S, Wang C. 2017. Evaluation of glutathione-binding protein A of *Haemophilus parasuis* as a vaccine candidate in a mouse model. *J Vet Med Sci* 79:184–187. <https://doi.org/10.1292/jvms.16-0327>.
 33. Zhang Y, Li Y, Yuan W, Xia Y, Shen Y. 2016. Autophagy is associated with pathogenesis of *Haemophilus parasuis*. *Front Microbiol* 7:1423. <https://doi.org/10.3389/fmicb.2016.01423>.
 34. Morozumi T, Nicolet J. 1986. Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. *J Clin Microbiol* 23:1022–1025.
 35. Turni C, Blackall PJ. 2005. Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis*. *Vet Microbiol* 106:145–151. <https://doi.org/10.1016/j.vetmic.2004.12.019>.