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Short communication

Characterization of serotypes and virulence genes of *Haemophilus parasuis* isolates from Central Vietnam

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ABSTRACT

Haemophilus parasuis is a commensal Gram-negative bacterial pathogen in the upper respiratory tract of pigs, which causes Glässer's disease. More than 15 serotypes of *H. parasuis* have been identified with apparent differences in virulence. In this research, we surveyed the prevalence and distribution of serotypes and known virulence genes of the *H. parasuis* isolates collected from sick and healthy pigs in Quang Binh and Thua Thien Hue provinces in Central Vietnam. By using bacterial isolation and polymerase chain reaction (PCR), 56 out of 814 (6.9%) samples were positive for *H. parasuis*. The most prevalent serotypes were serotype 5 (15/56, 26.8%), followed by serotype 2 (13/56, 23.2%) and serotype 4 (10/56, 17.9%). The *vta1* was the most frequently detected virulence gene which was present in 62.5% of the strains, followed by *vta3* (42.9%), *vta2* (39.3%), *HPM-1371* (35.7%), *capD* (30.4%), *HPM-1372* (12.5%), *lsgB* and *HPM-1373* (both shared 8.9%). Strong correlations between some serotypes and known virulence genes were observed, in which virulence genes *HPM-1371, HPM-1372, vta3, vta2* and *capD* were mainly clustered in serotypes 5/12, and *vta2* clustered in serotype 2. This study presents the first baseline information on the epidemiological characteristics of *H. parasuis* isolates from Central Vietnam.

1. Introduction

Haemophilus parasuis is a Gram-negative bacterium which causes Glässer's disease in pigs and leads to huge economic losses in the pig industry worldwide (Bouchet et al., 2008; Oliveira and Pijoan, 2004). The prevalence difference of more than 15 serotypes of *H. parasuis* in different geographical regions has been identified by several methods (Castilla et al., 2012; Gou et al., 2018; Wang et al., 2017). Using a one-step multiplex PCR method which is faster, more sensitive and specific than indirect hemagglutination test (IHA), all serotypes of *H. parasuis* strains can be classified except between serotypes 5 and 12 (Howell et al., 2015). The cross protection of vaccines against different serotypes is limited. Thus, it is of great importance to know the prevalence of different serotypes of *H. parasuis* in a region in order to effectively control Glässer's disease.

A number of virulence factors and virulence associated genes of *H. parasuis* have been identified previously (Xu et al., 2013; Zhou et al., 2012). The genes *lsgB*, *capD*, and *wza* which encode sialyltransferase, polysaccharide biosynthesis protein, and polysaccharide export protein respectively (Martinez-Moliner et al., 2012; Wang et al., 2013), the glycosyltransferases-encoding genes *HPM-1370*, *HPM-1371*, *HPM-1372, HPM-1373* (Lawrence and Bey, 2015), and the virulence-associated autotransporters-encoding genes *vta1*, *vta2*, and *vta3* (Olvera et al., 2012) have been reported to be related in several stages of pathogenesis of *H. parasuis* (Lawrence and Bey, 2015). *H. parasuis* isolates belonging to different serotypes may carry distinct patterns of virulence genes (Boerlin et al., 2013). So, detecting the presence of these virulence genes in the field strains and their correlation with serotypes may be useful in predicting the virulence of *H. parasuis* isolates. However, there is so far not a comprehensive research investigating the correlation

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between virulence genes and all serotypes of *H. parasuis* strains.

So far, there is still a lack of sufficient studies concerning *H. parasuis* and Glässer's disease in Vietnam. To obtain more information about the epidemiology of porcine *H. parasuis* infection, we isolated a total of 56 strains of *H. parasuis* from sick and healthy pigs in Quang Binh and Thua Thien Hue provinces in Central Vietnam, and analyzed their serotypes distribution and virulence genes profile. Moreover, the correlation between serotypes and known virulence genes of the isolates was also investigated.

2. Materials and methods

2.1. Sample collection

A total of 814 samples were taken from production farms (n = 10) and slaughterhouses (n = 10) localized in Quang Binh and Thua Thien Hue provinces (Central Vietnam) from June to September, 2017. In farms, nasal swabs were collected from pigs with and without clinical signs of Glässer's disease; in slaughterhouses, nasal swabs and lung tissues were collected from different pigs. The clinical signs of Glässer's disease as described by Rapp-Gabrielson (1999) include polyarthritis, cough, dyspnea, fever, and cyanosis were used as a criterion to identifying pigs with clinical signs. Nasal swab collected was carefully put into a labeled tube containing 2 ml brain heart infusion (BHI) broth. For lung specimen, a piece of the lung was carefully cut using sterile scissors, and transferred into a sterile re-closable zipper plastic bag. All samples collected were stored in a cool box and delivered to the lab for strain isolation within 24 h.

2.2. Isolation and identification of bacteria

The bacteria were isolated following methods described previously (Moller et al., 1993; Morozumi and Nicolet, 1986). The isolates were all identified as *H. parasuis* in accordance with their colony characteristic (Morozumi and Nicolet, 1986), nicotinamide adenine dinucleotide (NAD)-dependence (Moller et al., 1993), and 16S rRNA sequence identification (Oliveira et al., 2001). Briefly, the samples were plated on tryptic soy agar (TSA) (Difco, Detroit, MI, USA) containing 10 μg/ml of NAD (Sigma, St. Louis, MO, USA) and 5% of bovine serum. The plates were incubated at 37 °C for 36–48 h. All suspect colonies of *H. parasuis* were identified by using PCR to amplify the 16S rRNA gene followed by sequencing (16S rRNA-F: 5′-GTGATGAGGAAGGGTGGTGT-3′ and 16S rRNA-R: 5′-GGCTTCGTCACCCTCTGT-3′) (Oliveira et al., 2001). Positive colonies in PCR assay were transferred to 2 ml BHI broth (Sigma, St. Louis, MO, USA) and grown at 37 °C for 18–24 h. All strains were maintained at −80 °C until analysis.

2.3. Serotyping

The serotypes of all the field strains were identified by using a one-step multiplex PCR (mPCR) method as described by Howell et al. (2015). Briefly, a loopful of the bacteria from a pure culture plate was resuspended in 30 μl of ultrapure H_2O (CW biotech, Beijing, China) and lysis was carried out by digestion with proteinase K (Omega bio-tek, GA, USA) for 1 h at 56 °C. The mixture was boiled for 30 min, followed by cooling on ice, and the supernatant collected after centrifugation at 12,000 rpm for 3 min. The concentrations of the genomic DNA extracted for the performance of PCRs were measured by using Nanodrop-ND2000 system (Thermo Scientific, Waltham, UK). A 25 μl aliquot of reaction mixture including 12.5 μl of 2 × Taq Quick-Load master mix (CW biotech, Beijing, China), 3 μl of the primer mix (at 50 μM), 2 μl of the gDNA (conc. > 10 ng/μl) from the boiled supernatant, 0.25 μl of DMSO, and 7.25 μl of ultrapure H_2O was subject to PCR using the following program: initial denaturation at 94 °C for 30 s; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min, and a final extension of 68 °C for 5 min. The amplified products were analyzed by electrophoresis with 2% agarose gels.

Table 1
List of primers using to amplify known virulence genes (Lawrence and Bey, 2015).

Genes	Primers	Sequence (5′ → 3′)	Product size (bp)
lsgB	lsgB-F	ATGAATTTGATTATTTGTATGACTCCATTTC	969
	lsgB-R	CTATTGGCATGTGTAGTCAATTACTTC	
capD	capD-F	ATGTTAATGCCATTAATTTATTCATTG	780
	capD-R	TCGAACCGATAGAACCAGCAGCACCAGTC	
wza	wza-F	ATGTGTAAGTTAACTAAAGCTCTTG	840
	wza-R	AGCAATTGCTTCGGTTAACGTCATAC	
1370	1370-F	ATGCTAAAAAGAGTGTTTGATATTTTC	540
	1370-R	TATATTATGATTAACATAATC	
1371	1371-F	ATGAACTTTCTACCATTCGCCCTTCCCG	520
	1371-R	ATTATATTTGAATCCAGGTTCAATG	
1372	1372-F	ATGAAATTGTCTGTCTTAATGGCTGT	720
	1372-R	TCCGCCAAATGTACATCATCAC	
1373	1373-F	ATGAAATTGTCTGTCTTAATGGCTGT	462
	1373-R	CTCTCATACCATACCCCAACTCAGG	
vta 1	vta1-F	TTTAGGTAAAGATAAGCAAGGAAATCC	406
	vta1-R	CCACACAAAACCTACCCCTCCTCC	
vta 2	vta2-F	AGCTTATATTCTCAGCACAAGGTGC	294
	vta2-R	CCACTGATAACCTACCCCCACAGAG	
vta 3	vta3-F	AATGGTAGCCAGTTGTATAATGTTGC	293
	vta3-R	CCACTGTAATGCAATACCTGCACC	

2.4. Virulence gene identification

The presence of each virulence gene in the *H. parasuis* strains was determined by using a simple PCR method as described by Lawrence and Bey (2015). The known virulence genes, primers and the sizes of each amplified product were showed in Table 1. The PCR mixture (total volume 25 μl) included 12.5 μl of 2 x Taq PCR master mix (CW biotech, Beijing, China), 2 μl of each primer (0.2 mM), 7.5 μl of ultrapure H_2O, and 1 μl of gDNA (conc. > 10 ng/μl) for each isolate. All PCR products were analyzed by electrophoresis with 1% agarose gels.

2.5. Data analysis

The software SPSS (IBM SPSS Statistics version 18.0, IBM, Armonk, NY, USA) was used for statistical analysis. The pairwise comparison of positive isolation rates between regions where samples were collected, specimen origins, age, with or without clinical appearance, and difference in virulence genes were performed by *Chi*-squared test. All tests were 2-sided, and *p* values lower than 0.05 were defined as significantly different. Associations between serotypes, characteristic serotypes and known virulence genes were calculated using *Chi*-square and Fisher's extract tests. Statistically significant associations were shown as odds ratios (OR) with 95% confidence interval (CI) and when the *p* value was lower than 0.05.

3. Results and discussion

3.1. Prevalence of H. parasuis strains

A total of 56 strains of *H. parasuis* were isolated and identified from 814 samples in pigs from farms and slaughterhouses in Quang Binh and Thua Thien Hue provinces, Central Vietnam (Table 2). Previous studies have reported that the isolation rate of *H. parasuis* varied from 0.99% to 54.0% depending on different geographical regions (Cu et al., 2005; Fablet et al., 2012; Wang et al., 2017; Zhang et al., 2012). In this study, the results revealed that the isolation rate of *H. parasuis* varied with regions where samples were taken, origins of specimens, and ages, with significant difference ($p < 0.05$). The positive detection rate of *H. parasuis* in piglets (12/124, 9.7%) was significantly higher than finishing pigs (6/172, 3.5%) ($p = 0.02$), which was consistent with a

Table 2
H. parasuis infections in Quang Binh and Thua Thien Hue provinces, Vietnam.

			Quang Binh province			Thua Thien Hue province			**Total**		
			No. samples	Positive	%	No. samples	Positive	%	No. samples	Positive	%
Slaughterhouse		Lung	106	5	4.7	164	25	15.2	270	31	11.5[a]
		Nasal swab	140	0	0.0	108	8	7.4	248	7	2.8
		Subtotal	246	5	2.0	272	33	12.1	518	38	7.34[c]
Farm	Age groups	Piglet	68	7	10.3	56	5	8.9	124	12	9.7[b]
		Finishing pig	72	6	8.3	100	0	0.0	172	6	3.5
	Clinical sign	With (sick)	70	10	14.3	83	3	3.6	153	13	8.5[c]
		Without (healthy)	70	3	4.3	73	2	2.7	143	5	3.5
	Subtotal		140	13	9.3	156	5	3.2	296	18	6.1
Total			386	18	4.7[d]	428	38	8.9	814	56	6.9

[a] Show a signification difference isolation frequency among lung and swab samples $p < 0.001$.
[b] Shown a signification difference isolation frequency among ages group $p < 0.05$.
[c] Shown no signification difference isolation frequency among farm and slaughterhouses, with or without clinical $p > 0.05$.
[d] Show a signification difference isolation frequency among difference regions $p < 0.05$.

previous report (Wang et al., 2017), reconfirming that *H. parasuis* infection was a larger threat to piglets than to finishing pigs. In most of the earlier studies (Cai et al., 2005; Rúbies et al., 1999; Zhang et al., 2012) *H. parasuis* were mainly isolated from brain, lung, joints, and pleura specimens. A total of 183/818 (22.1%) *H. parasuis* strains were isolated from lung, brain, and join of pigs in China (Cai et al., 2005) and 174/327 (53.2%) strains were isolated from lung, pericardial and ascetic fluids of pigs in Spain (Rúbies et al., 1999). In this study, the isolation rate of *H. parasuis* was found to be higher in lung specimens (11.5%, 31/270) than in nasal swabs (2.8%, 7/248) ($p = 0.0002$).

3.2. Distribution of H. parasuis serotypes

Nine serotypes were identified in the 56 *H. parasuis* strains. Serotypes 5 (26.8%, 15/56) was most commonly identified, followed by serotype 2 (23.2%), serotype 4 (17.9%), serotype 10 (12.5%), and serotype 9 (10.7%); whereas serotypes 1, 6, 7, and 8 were only represented by a small number of strains, and serotypes 3, 11, 13, 14, 15 were not found (Fig. 1D). The detection frequency of serotype 5 (13/38, 34.2%) from pigs in Thua Thien Hue was higher than that in Quang Binh (2/18, 11.1%) ($p < 0.05$). In contrast, the detection frequency of serotype 4 in Thua Thien Hue was lower than that in Quang Binh ($p = 0.03$) (Fig. 1A). The detection frequency of serotype 5 from lung specimens was higher than that from nasal swabs ($p = 0.0005$) (Fig. 1B). The prevalence pattern of serotypes in this study is similar to previous studies in China (Cai et al., 2005), Italy (Luppi et al., 2013), Germany (Brogden et al., 2018), and North Vietnam (Cu et al., 2005). In addition, the detection frequency of serotypes 2, 9, and 10 was slightly higher than that reported in previous studies (Jia et al., 2017; Ma et al., 2016). In contrast to previous studies which reported that serotypes 3, 11, 13, 14, and 15 were found, among which serotype 13 was the most prevalent (Cai et al., 2005; Ma et al., 2016), we did not isolate any strains belonging to these serotypes. Strains of serotype 4 (5/13), serotype 2 (3/13), serotype 10 (2/13), serotype 1 (1/13), serotype 6 (1/13) and serotype 9 (1/13) could be isolated from sick pigs with clinical signs, while serotype 2 (2/5), serotype 9 (2/5) and serotype 5/12 (1/5) was

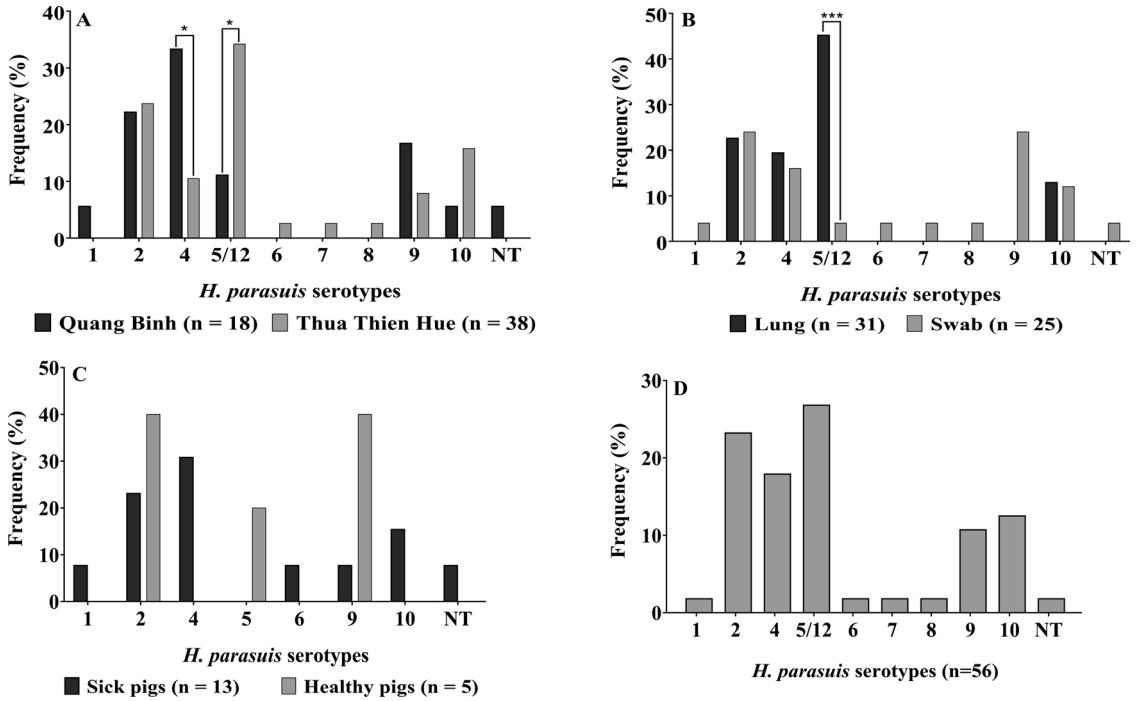

Fig. 1. Serotype prevalence of *H. parasuis* isolates. Distribution of different serotypes of *H. parasuis* isolates in difference provinces (A), in difference samples (B), from sick and healthy pigs (C), and in all isolates (D). NT, Non-serotypeable. Statistically significant values ($p < 0.05$) were indicated by asterisks.

Table 3
Distribution virulence genes among serotypes or characteristic serotypes of *H. parasuis* strains.

		Virulence gene frequencies									
	No. strains	*lsgB*	*capD*	*wza*	*1371*	*1372*	*1373*	*vta1*	*vta2*	*vta3*	None
Total	56	5	17	3	20	7	5	35	22	24	7
Serotype 1	1		1		1					1	
Serotype 2	13	1	3		1			6	9	7	
Serotype 4	11	1	2		2	1	2	9	3	3	
Serotype 5/12	14	2	9	3	11	5	2	12	10	11	
Serotype 6	1										1
Serotype 7	1				1						
Serotype 8	1										1
Serotype 9	6							2			4
Serotype 10	7	1	2		4	1	1	6		2	
Non-typeable	1										1
Highly virulence[a]	22	3	12	3	16	6	3	18	10	14	
Moderately virulence[a]	24	2	5		3	1	2	15	12	10	
Non-virulence[a]	9				1			2			6

[a] Serotypes used as an indicator of virulence are according to described by Oliveira and Pijoan (2004).

isolated from the healthy pigs (Fig. 1C).

3.3. Virulence gene analysis of the H. parasuis strains

The data in Table 3 and Fig. 2 revealed that the *H. parasuis* strains of different serotypes or origins possessed different virulence genes. The *vta1* was identified in most of the strains (35/56), followed by *vta3* (24/56), *vta2* (22/56), *HPM-1371* (20/56), *capD* (17/56), *HPM-1372* (7/56), *lsgB* and *HPM-1373* (both shared 5/56), and *wza* (3/56). The strains isolated from lung specimens possessed the most known virulence genes (Fig. 2A). A significant difference in the distribution of *HPM-1371* and *vta1*, *vta2, vta3* was observed between strains collected from lungs and nasal swabs ($p < 0.05$). The frequency of *HPM-1371* in *H. parasuis* strains from Thua Thien Hue (17/56) was higher than that from Quang Binh (3/56) with significance ($p = 0.04$) (Fig. 2B). We have also compared the distribution of virulence genes *lsgB*, *capD*, *HMP-1372*, *vta1*, and *vta3* in pigs with and without clinical signs. However, as shown in Fig. 2C, no significant difference was observed between the isolates from sick and healthy pigs ($p > 0.05$). This may be due to our limited understanding of the virulence genes of this pathogen.

The previous studies showed that *vta3* was highly conserved among virulent and non-virulent strains, while *vta1* and *vta2* were mainly identified in virulent strains (Pina et al., 2009). In addition, Olvera et al. (2012) reported that non-virulent strains were positive in all of the

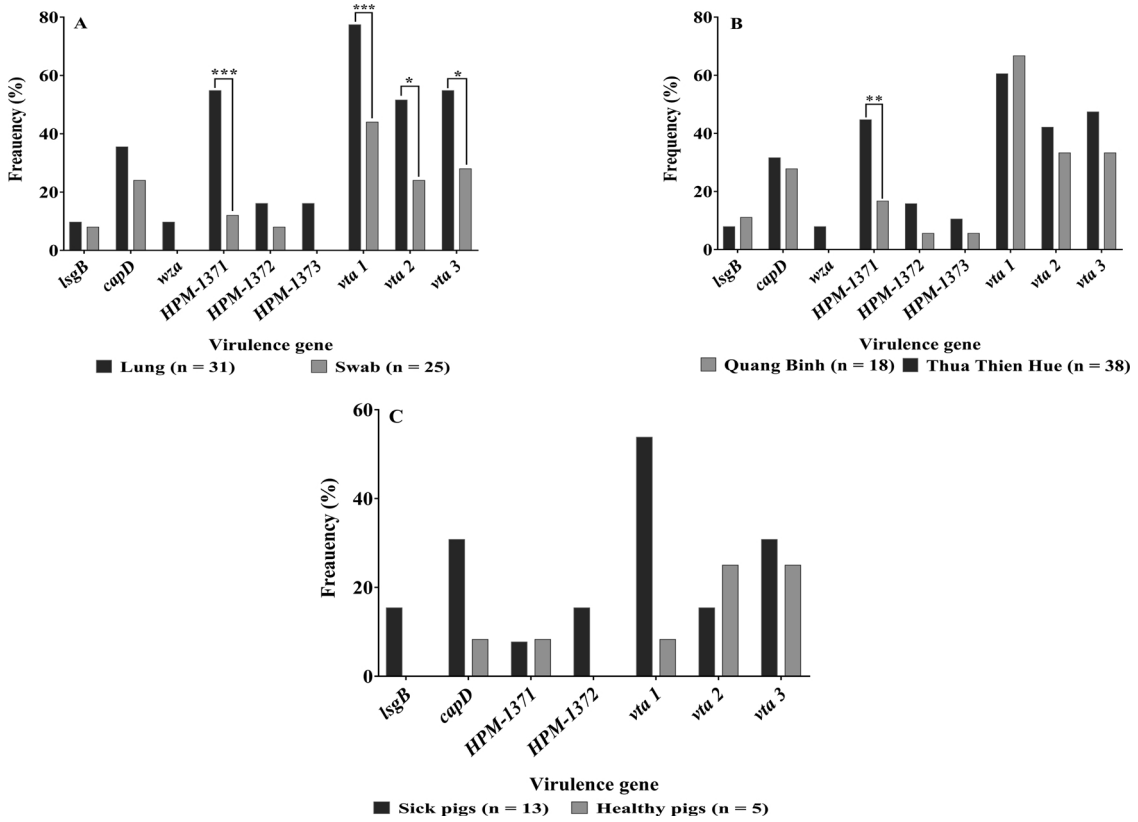

Fig. 2. Distribution of known virulence genes in *H. parasuis* isolates. Distribution of known virulence genes in the isolates from different samples (A), from different regions (B), and from sick and healthy pigs (C). Statistically significant values ($p < 0.05$) were indicated by asterisks.

three *vta* genes, whereas, the highly, moderately, and mildly virulent strains were only positive in *vta1* and *vta3*. In this present study, the detection frequencies of *vta1*, *vta2* and *vta3* in strains isolated from lung specimens were higher than that isolated from nasal swabs ($p < 0.05$) (Fig. 2A). The results herein were similar to those in previous studies (Lawrence and Bey, 2015; Olvera et al., 2012). The sialyltransferase encoding gene *lsgB* was reported to be found only in virulent strains (Martinez-Moliner et al., 2012). The result in this study showed that 5/56 strains belonging to the highly and moderately virulent were positive for *lsgB*, in which 3/5 strains were isolated from lung specimens. The *capD* gene encodes a polysaccharide biosynthesis protein that is related to the virulence of *H. parasuis* (Zhou et al., 2010). In the present study, 17/56 (30.36%) strains were positive for *capD* and all of them were highly and moderately virulent, which was similar to the results reported in the previous studies (Wang et al., 2013; Zhou et al., 2010). The functions of the conserved *wza-wzb-wzc* genes in *Escherichia coli* have been identified previously (Reid and Whitfield, 2005). In our study, only 3/56 strains were positive for *wza* gene and all of them were highly virulent strains. The presence or absence of the glycosyltransferases was related to the immunogenicity of pathogenic bacteria and the bacterium avoiding phagocytosis (Lawrence and Bey, 2015). The results in this study showed that, the *H. parasuis* strains were positive to the four glycosyltransferases *HPM-1370, HPM-1371, HPM-1372*, and *HPM-1373* (Table 3). Lawrence and Bey (2015) have reported the *H. parasuis* serotypes 4 and 5 lacking the polysaccharide export protein, which exposes a functional overlap among ABC transporters in exporting polysaccharide polymers. Our results showed that *H. parasuis* strains of the multifarious genotypes were in different frequencies, confirming the complex epidemiology of *H. parasuis* in pigs.

3.4. Association between serotypes, characteristic serotypes and virulence genes

One strain of serotype 6 and serotype 8 each, four strains of serotype 9, and one non-typeable strain did not carry any known virulence genes, and strains of all the serotypes carried at least one virulence gene (Table 3). A strong significant correlation was observed between serotype 2, serotype 5/12 and the presence of some virulence genes. A significant association was observed between serotype 2 and *HPM-1371* (OR = 0.1, $p = 0.02$), *vta2* (OR = 5.2, $p = 0.02$). Serotype 5/12 was associated with *capD* (OR = 7.7, $p = 0.003$), *HPM-1371* (OR = 13.4, $p = 0.0002$), *HPM-1372* (OR = 11.1, $p = 0.01$), *vta2* (OR = 6.3, $p = 0.01$), *vta3* (OR = 8.2, $p = 0.004$) (Table 4). According to a previous review by Oliveira and Pijoan (2004) *H. parasuis* of different serotypes were classified of into three groups: highly virulent (serotypes 1, 5, 10, 12, 13 and 14), moderately virulent (serotypes 2, 4, and 15) and non-virulent (serotypes 3, 6, 7, 8, 9, and 11). In this study, the virulence genes *lsgB*, *capD*, *wza*, *HPM-1372*, *HPM-1373*, *vta2*, and *vta3* were not found in any strains belonging to the non-virulent group. Most of the virulence genes were distributed in the highly and moderately virulent groups (Table 3). Significantly increased probability of the presence of HPM-*1371* (OR = 20.0, $p < 0.0001$), *HPM-1372* (OR = 12.4, $p = 0.01$), *capD* (OR = 7.0, $p = 0.003$), and *vta1* (OR = 4.5, $p = 0.02$) was found in the highly virulent strains. In contrast, significantly decreased probability of the presence of HPM-1371 (OR = 0.1, p = 0.002) and vta1 (OR = 0.1, p = 0.01) was found in the moderately and non-virulent strains, respectively (Table 4). Thus, the combination of multiple virulence genes may work together to confer a relatively higher virulence in H. parasuis strains. The serotyping scheme for *H. parasuis* based only on the presence of clinical signs and presence of bacteria in some specimens as reported by (Oliveira and Pijoan, 2004) was not exact to decide the virulence. The results in a study by Yu et al. (2014) were not completely consistent with the serotyping scheme, which revealed that some strains belonging to the same serotype showed different virulence. Moreover, according to our results in this study, some strains belonging to the same virulence group (serotypes 2 and 5) carried different virulence genes, in which the strains of serotype 2 were only positive associated with *vta2*, while the strains of serotype 5 were positive associated with five virulence genes. Therefore, our study suggests that virulence genes detection may be a complementary approach for accurate arranging serotypes of *H. parasuis* into different virulence groups.

4. Conclusion

In this study, we investigated the prevalence of *H. parasuis* in pigs in Quang Binh and Thua Thien Hue province, Central Vietnam, and analyzed the serotypes and the presence of putative virulence genes of the isolates. The results herein provided the first baseline information on the serotype characteristics and virulence genes of *H. parasuis* in Central Vietnam, which are important for understanding the epidemiological characteristics and the development of vaccination used in preventing the infection caused by *H. parasuis* in pig.

Table 4
Associations between serotypes or characteristic serotypes and virulence genes of *H. parasuis* strains.

Serotypes orcharacteristic serotypes	Characteristics of strains					Agreement between serotypes or characteristic serotypes and VGs (n = 56)		
	n-Pr[a]	VGs	n-Gp[b]	P+/G−[c]	P−/G+[d]	OR	95% CI	*p*
Serotype 2	13	*1371*	20	12	19	0.1	0–0.9	0.02
		vta2	22	4	13	5.2	1.4–19.9	0.02
Serotype 5/12	14	*capD*	17	5	8	7.7	2–29.1	0.003
		1371	20	3	9	13.4	3.1–58.7	0.0002
		1372	7	9	2	11.1	1.9–66.7	0.01
		vta2	22	4	12	6.3	1.6–23.8	0.01
		vta3	24	3	13	8.2	1.9–34.3	0.004
Highly virulence	22	*capD*	17	10	5	7.0	2–24.7	0.003
		1371	20	6	4	20.0	4.9–81.4	< 0.0001
		1372	7	16	1	12.4	1.4–111.6	0.01
		vta1	24	8	10	4.2	1.3–13.1	0.02
Moderately virulence	24	*1371*	20	21	17	0.1	0–0.5	0.002
Non-virulence	9	*vta1*	35	7	33	0.1	0–0.7	0.01

[a] n-Pr Number of strains in the serotype or characteristic of serotypes.
[b] n-Gp Number of strains positive with known virulence genes.
[c] P+/G− Number of strains in the serotype or characteristic of serotype, but no carrying known virulence gene.
[d] P−/G+ Number of strains no in the serotype or characteristic of serotype but carrying known virulence gene.

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