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RESEARCH ARTICLE

Association Between Virulence Genes and Serovars, Sequence Types of *Glaesserella (Haemophilus) parasuis* Isolates from the Nasal Cavity of Live Piglets

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Abstract: This study analyzed the 19 virulence genes (VGs) of 117 *Glaesserella (Haemophilus) parasuis (G. parasuis)* isolates from the nasal cavities of live piglets from the south of China and assessed the associations between VGs and serovars, sequence types (STs) of these isolates. The detection rate of 19 VGs ranged from 1.7% to 95.2%, with *vacJ* and *clpP* (95.7%) as the most prevalent. Of the 117 *G. parasuis* isolates, 105 were assigned to ten distinct serovars (1, 2, 4-10 and 15), and twelve of the isolates tested were non-typable (NT). The serovar 10 (17.9%) was the most prevalent. The *G. parasuis* isolates belonging to the same ST and serovar harbored different VGs, and all isolates exhibited considerable genetic heterogeneity. Significant correlations were found between VGs and serovars, different pathogenic serovar groups, and members of clade 2 (based on ST). The results complement epidemiological data of *G. parasuis* and will help the scientific community understand the extreme genetic diversity and pathogenesis of *G. parasuis,* which will aid in the development of *G. parasuis* vaccines.

Keywords: *Glaesserella (Haemophilus) parasuis, Virulence gene, Serovar,* Sequence type*, Live piglet*

Canlı Domuz Yavrularının Burun Boşluğundan İzole Edilen *Glaesserella (Haemophilus) parasuis*'in Virülans Genleri İle Serovar ve Sekans Tipleri Arasındaki İlişki

Öz: Bu çalışmada, Çin'in güneyinde canlı domuz yavrularının burun boşluklarından elde edilen 117 *Glaesserella (Haemophilus) parasuis (G. parasuis)* izolatının 19 virülans geni (VG'ler) analiz edildi ve VG'ler ile serovarlar ve sekans tipleri (ST'ler) arasındaki ilişki değerlendirdi. 19 VG'nin pozitiflik oranı %1.7 ile %95.2 arasında değişmekte olup, en yaygın (%95.7) *vacJ* ve *clpP* genleri saptandı. 117 *G. parasuis* izolatının 105'i on farklı serovar (1, 2, 4-10 ve 15) içerisinde yer alırken, test edilen izolatlardan 12'si serotiplendirilemedi (NT). Serovar 10 (%17.9) en yaygın olanıydı. Aynı sekans tipi ve serovara ait olan *G. parasuis* izolatları farklı VG'ler barındırır iken, tüm izolatlar önemli ölçüde genetik heterojenite sergiledi. VG'ler ile serovarlar, farklı patojenik serovar grupları ve ST tabanlı monofiletik grup 2 (klad 2) üyeleri arasında önemli korelasyonlar saptandı. Bulgular, *G. parasuis*'in epidemiyolojik özelliklerini tamamlamakta olup, bilim camiasına, *G. parasuis* etkenine karşı aşı geliştirilmesine katkı sağlayacak geniş genetik çeşitliliğinin ve patogenezinin aydınlatılması yönünde yardımcı olacaktır.

Anahtar sözcükler: *Glaesserella (Haemophilus) parasuis, Virülans gen, Serovar, Sekans tipi, Canlı domuz yavrusu*

INTRODUCTION

Glaesserella (Haemophilus) parasuis (G. parasuis), the pathogen that causes Glässer's disease, has brought huge economic losses to the global swine industry [1,2]. *G. parasuis* is a commensal bacterium in the swine upper respiratory tract that contains strains ranging from non-virulent to highly virulent. Virulent strains can invade and cause systemic disease under certain conditions [3-5].

To date, 15 serovars have been identified, in addition to some non-typable (NT) strains [6,7]. Serovar identification of the isolates is the basis for designing vaccination programs [8]. Some earlier studies suggested that *G. parasuis* serovars were virulence markers and could be divided into three pathogenic groups [2]. However, later studies found that isolates allocated into non-pathogenic serovars can also cause disease, and virulence of the isolates allocated to the same serovar can vary greatly [9-11]. Thus, it remains

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unclear whether serovar can be used as a marker of virulence in *G. parasuis*.

It is generally believed that a single virulence gene (VG) may not be a decisive factor in triggering the pathogenesis of multifactorial diseases such as Glässer's disease, and the pathogenesis of bacteria often depends on the interaction and expression regulation of many VGs. Thus, a comprehensive analysis of VGs in clinical isolates may be helpful to predict the pathogenicity of novel *G. parasuis* isolates as they are identified. Although the characteristics of *G. parasuis* isolates from clinical cases have been extensively studied, an in-depth analysis of *G. parasuis* isolates from the swine upper respiratory tract has not been performed. In this study, we analyzed the characteristics, including serovars and VGs, of *G. parasuis* isolates from the nasal cavities of live piglets in the south of China. Our results provide more information on the epidemiology and pathogenesis of *G. parasuis*.

Material and Methods

Identification and Serotyping

Nasal swabs were collected from the nasal cavities of live piglets without obvious clinical symptoms of Glässer's disease between 2007 and 2016 in three provinces (Guangdong, Jiangxi, and Shanghai) in the south of China. Nasal swabs were inoculated on blood agar medium with 0.0025% of NAD immediately after sampling. Suspect *G. parasuis* colonies were identified by NAD-dependency and 16S rRNA PCR [12]. The isolates underwent molecular serotyping via a multiplex PCR assay described in Howell et al.[13].

Table 1. *Primers used to amplify VGs*

VGs	Primers	Sequence (5'→3')	Product Size
hhdA	*hhdAF*	GGTTCTAGTTCACAAACAGCCAATAC	964
	hhdAR	GATATTTACCCCTGCCTTCATTGTATC	
hhdB	*hhdBF*	ATCTTGCCCTGATTAGAGAGTAGGAGT	557
	hhdBR	GTGAATATAGCCCTTATCCAAATAGGC	
fhuA	*fhuAF*	ATGGTTTGGTTGTAATGGAGTATC	563
	fhuAR	AACAACGCCAGCTAGGCTTGTACT	
vta1	*vta1F*	TTTAGGTAAAGATAAGCAAGGAAATCC	406
	vta1R	CCACACAAAACCTACCCCTCCTCC	
wbgY	*wbgYF*	TTAGGGCTTGTCGCCCTATTTC	380
	wbgYR	GAAGCACTATCTGTAATACCAGGC	
fimB	*fimBF*	CTAAGAGAGAGCAGGGCGATAGAA	386
	fimBR	TGTCACCACAATGGCTCAGGTTGA	
hsdR	*hsdRF*	GCAAGCTTACTCTCGTACTAACCG	410
	hsdRR	AGGCTCCACTAGGTTCTTCTACTC	
nhaC	*nhaCF*	CATATTGTGGTACAAGGTGGCGAG	415
	nhaCR	CTAATACGGAAGTCACTGTACCGC	
H0254	*H0254F*	CAGTGAAAGTCGTGATGTGGAACC	397
	H0254R	GGACGTTCGTTCACATCTTGTTCG	
capD	*capDF*	CGAAGGGAGTGTTTCTATCA	958
	capDR	GAGTTTCTCACCAGGTCTAA	
rfaE	*rfaEF*	GCAGGGCGAGCGTTGGATAA	524
	rfaER	TGGGTCGGTAAATGGAATGG	
lsgB	*lsgBF*	ATGAATTTGATTATTTGTATGACTCCATTT	969
	lsgBR	CTATTGGCATGTGTAGTCAATTACTTC	
HPM1370	*HPM1370F*	ATGCTAAAAAGAGTGTTTGATATTTTC	540
	HPM1370R	TATATTATGATTAACATAATC	
HPM1371	*HPM1371F*	ATGAACTTTCTACCATTCGCCCTTCCCG	520
	HPM1371R	ATTATATTTGAATCCAGGTTCAATG	
HPM1372	*HPM1372F*	ATGAAATTGTCTGTCTTAATGGCTGT	720
	HPM1372R	TCCGCCAAATGTACATCATCAC	
HPM1373	*HPM1373F*	ATGAAATTGTCTGTCTTAATGGCTGT	462
	HPM1373R	CTCTCATACCATACCCCAACTCAGG	
clpP	*clpPF*	AGAGTGAGGGCGTTGAGT	331
	clpPR	TTCTTGTTTCGGGTGTTT	
cheY	*cheYF*	CCTTATGATGCCGTAGTTCTCG	443
	cheYR	TCAAGAGCGTTGCTACTGACCT	
vacJ	*vacJF*	ACCGTGCCATGTGGAAAGTC	377
	vacJR	TAAATCTTGACGAGGCGTTGC	

VG Analysis

Nineteen VGs were analyzed using PCR as previously described [14-23]. Details of all primers used are listed in *Table 1*.

Sequence Types (STs) Analysis

A STs analysis was carried out using the Multi-locus Sequence Typing (MLST) method as previously described [24,25]. A neighbor-joining tree was built using the MEGA version 5.0 software based on the MLST target sequences.

Statistical Analyses

Chi-square and Fisher's exact tests were used to assess the associations between serovars, ST, and VGs using SPSS version 18.0, and p values lower than 0.05 were considered statistically significant associations.

Results

Identification and Serotyping

A total of 117 *G. parasuis* isolates were obtained from 710 nasal swab samples. Of the 117 *G. parasuis* isolates, 105 were assigned to ten distinct serovars, and twelve of the isolates tested were NT. Serovar 10 (17.9%) was the most prevalent, followed by serovars 15 (14.5%), 6 (12.0%), 8 (11.1%), 4 (8.5%), 9 (7.7%), 1 (7.7%), 7 (6.0%), 5/12 (4.3%), and 2 (0.9%) *(Fig. 1-A)*. Serovars 3, 11, 13, and 14 were not identified. Serovars 4, 6, 15, and NT were observed in all three provinces. However, serovar 2 was observed only in Shanghai and serovar 7 was observed only in Jiangxi *(Fig. 1-B)*.

VG Analysis

The VGs *vacJ* and *clpP* (95.7%) were the most prevalent, followed by *cheY* (93.2%), *rfaE* (92.3%), *hsdR* (91.5%), *capD* (88.9%), *fhuA* (40.2%), *vta1* (35.9%), *hhdA* (33.3%), *hhdB* (26.5%), *HPM1372* (22.2%), *nhaC* (21.4%), *lsgB* (19.7%), *H0254* (10.3%), *fimB* (10.3%), *wbgY* (7.7%), *HPM1373* (6.8%), *HPM1371* (5.3%), *HPM1370* (1.7%) *(Fig. 2)*. All *G. parasuis* isolates were clustered according to the presence of VGs. Four clusters were obtained (clusters A, B, C, and D) *(Fig. 3)*. Cluster A includes serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and NT isolates, harboring 4 to 11 VGs; Cluster B includes serovars 4, 5/12, 6, and NT isolates, harboring 9 to 17 VGs; Cluster C includes serovars 1, 7, and 10, harboring 5 to 8 VGs; and Cluster D includes only NT isolates, harboring 0 to 4 VGs. Interestingly, some serovars were distributed in 2 or 3 clusters. For example, serovars 4 and 6 were found in clusters A and B, serovars 1, 7, and 10 were found in clusters A and C, and NT isolates were found in clusters A, B, and D *(Fig. 3)*.

Association Between Serovars and VGs

The distribution of VGs in the isolates allocated to different serovars varied greatly, and a significant correlation was found between serovars and some VGs. A significant

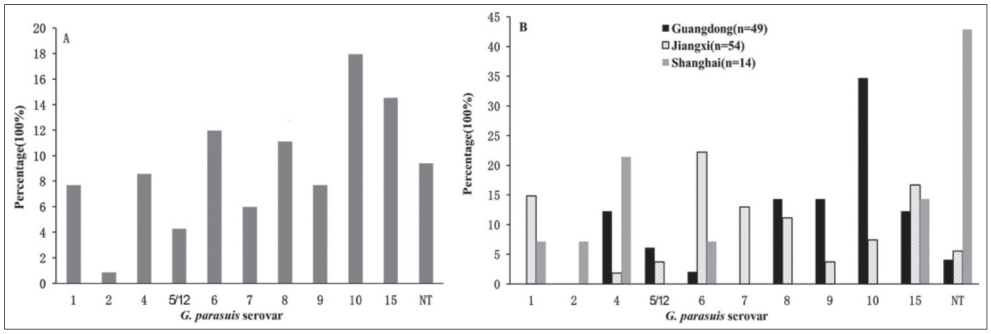
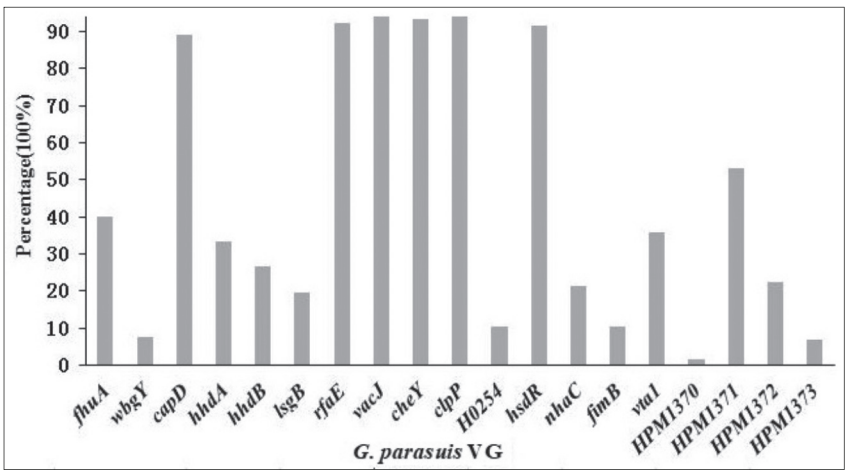


Fig 1. The distribution of serovar in all 117 isolates(A) and in difference provinces(B)

Fig 2. The distribution of 19 VGs in all 117 *G. parasuis* isolates



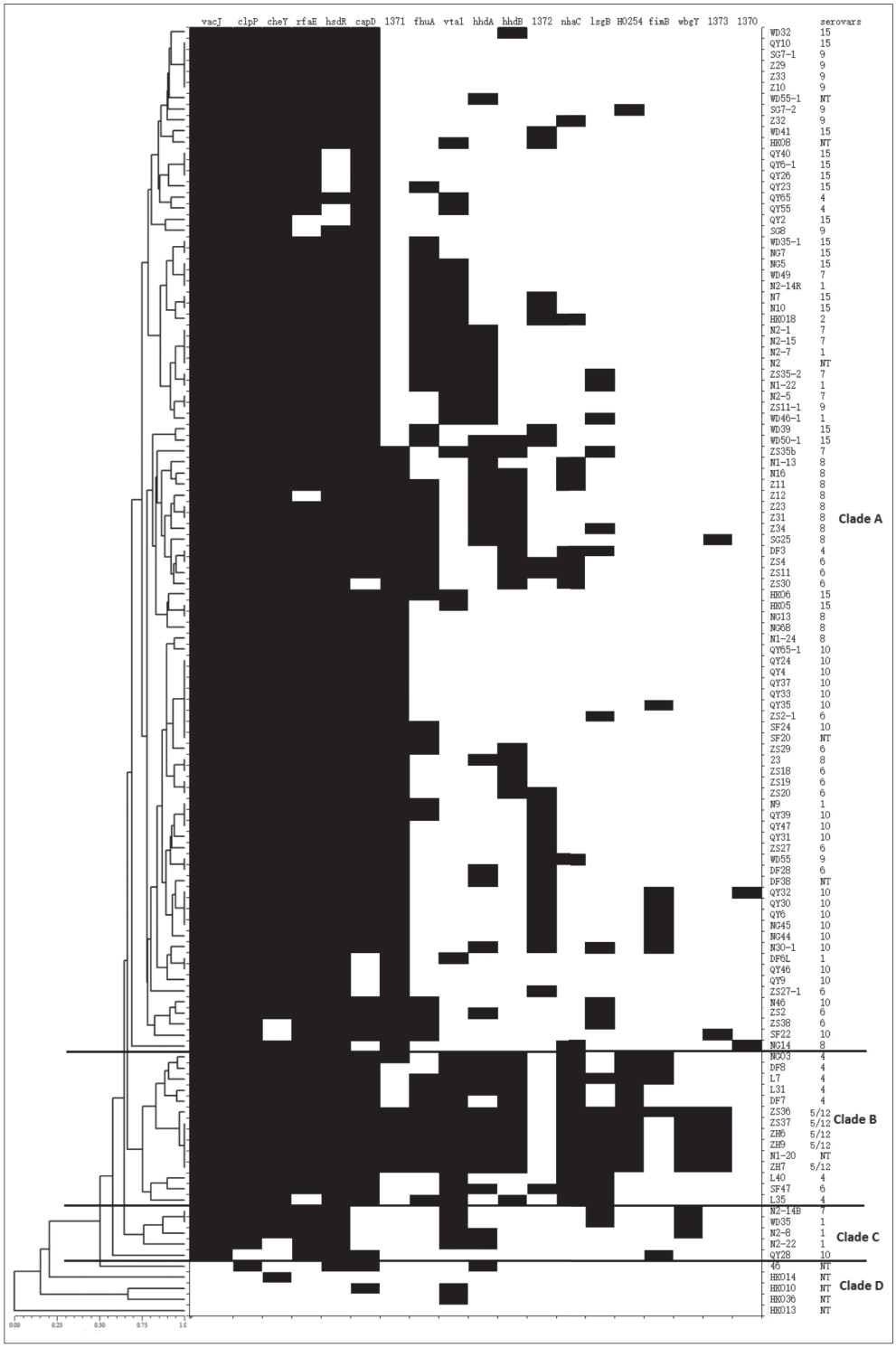


Fig 3. Clustering of *G. parasuis* isolates based on VGs

positive correlation was found between the following: serovar 1 and *vta1*; serovar 4 and *hhdB, H0254, nhaC,* and *vta1*; serovar 5/12 and *fhuA, wbgY, hhdA, hhdB, lsgB, H0254, nhaC, vta1,* and *HPM1373*; serovar 6 and both *HPM1371,* and *HPM1372*; serovar 7 and both *hhdA* and *vta1*; serovar 8 and *hhdA, hhdB,* and *HPM1371*; serovar 10 and *fimB, HPM1371,* and *HPM1372*; serovar 15 and *hsdR*. However, a significant negative correlation was found between serovar 1 and *capD*, serovar 4 and *HPM1371,* serovar 6 and *vta1*, serovar 8 and *vta1*, serovar 9 and both *fhuA* and *HPM1371*, and the following: serovar 10 and *fhuA*, *hhdA*, *hhdB*, *nhaC*, and *vta1*, serovar 15 and *hhdA*, *lsgB*, *nhaC*, and *HPM1371*, and NT and r*faE*, *vacJ*, *cheY*, *clpP*, and *hsdR* (P<0.05, *Table 2).*

Table 2. Association between serovars and VGs of G. parasuis isolates

Serovar	VGs	VG +	VG−	−VG +	−VG−	OR	95% CI	P
1	*capD*	5	4	99	9	0.11	0.03-0.48	0.009
	vta1	8	1	34	74	17.41	2.09-144.78	0.001
5/12	*fhuA*	5	0	42	70	∞	/	0.009
	wbgY	5	0	4	108	∞	/	0.000
	hhdA	5	0	34	78	∞	/	0.003
	hhdB	5	0	26	86	∞	/	0.001
	lsgB	5	0	18	94	∞	/	0.000201
	H0254	5	0	7	105	∞	/	0.000005
	nhaC	5	0	20	92	∞	/	0.000317
	vta1	5	0	37	75	∞	/	0.005
	HPM 1373	5	0	3	109	∞	/	0.000
10	*fhuA*	4	17	43	53	0.29	0.09-0.93	0.047
	hhdA	1	20	38	58	0.08	0.01-0.62	0.002
	hhdB	0	21	31	65	0	/	0.001
	nhaC	0	21	25	71	0	/	0.006
	fimB	8	13	4	92	14.15	3.73-53.68	0.000094
	vta1	0	21	42	54	0	/	0.000031
	HPM1371	20	1	42	54	25.71	3.31-199.41	0.000007
	HPM 1372	9	12	17	79	3.49	1.27-9.59	0.019
4	*hhdB*	7	3	24	83	8.07	1.94-33.61	0.003
	H0254	5	5	7	100	14.29	3.33-61.37	0.001
	nhaC	8	2	17	90	21.18	4.13-108.52	0.000053
	vta1	9	1	33	74	20.18	2.46-165.85	0.000391
	HPM 1371	2	8	60	47	0.2	0.04-0.99	0.044
15	*hhdA*	1	16	38	62	0.1	0.01-0.78	0.011
	lsgB	0	17	23	77	0	/	0.023
	hsdR	12	5	95	5	0.13	0.03-0.52	0.006
	nhaC	0	17	25	75	0	/	0.022
	HPM 1371	2	15	60	40	0.09	0.02-0.42	0.000336
8	*hhdA*	9	4	30	74	5.55	1.59-19.41	0.009
	hhdB	8	5	23	81	5.63	1.68-18.87	0.005
	vta1	0	13	42	62	0	/	0.004
	HPM 1371	13	0	49	55	∞	/	0.000158
6	*vta1*	1	13	41	62	0.12	0.02-0.95	0.017
	HPM 1371	13	1	49	54	14.33	1.81-113.61	0.001
	HPM 1372	7	7	19	84	4.42	1.39-14.10	0.014
7	*hhdA*	5	2	34	76	5.59	1.03-30.26	0.040
	vta1	7	0	35	75	∞	/	0.001
9	*fhuA*	0	9	47	61	0	/	0.011
	HPM 1371	1	8	61	47	0.1	0.01-0.83	0.012
NT	*rfaE*	6	5	102	4	0.05	0.01-0.24	0.000289
	vacJ	6	5	106	0	0	/	0.000003
	cheY	7	4	102	4	0.07	0.01-0.34	0.003
	clpP	7	4	105	1	0.02	0-0.20	0.000212
	hsdR	7	4	100	6	0.11	0/03-0.48	0.007

VG +: *Number of isolates in the corresponding serovar but carrying the VG;* ***VG−:*** *Number of isolates in the corresponding serovar but no carrying the VG*
−VG +: *Number of isolates no in the corresponding serovar but carrying VG;* ***−VG −:*** *Number of isolates no in the corresponding serovar but no carrying VG*

Table 3. *Association between pathogenic serovar group and VGs of G. parasuis isolates*

Pathogenic Serovar Group	VGs	VG +	VG−	−VG +	−VG−	OR	95% CI	P
Highly pathogenic group	*wbgY*	7	28	1	70	17.5	2.06-148.84	0.002
	hhdB	5	30	25	46	0.31	0.11-0.90	0.038
	fimB	9	26	3	68	7.85	1.97-31.28	0.002
	HPM 1371	27	8	32	39	4.11	1.64-10.28	0.002
	HPM 1373	6	29	1	70	14.48	1.67-125.66	0.005
Moderately pathogenic group	*hsdR*	22	6	78	0	0	/	0.0002
	vta1	15	13	22	56	2.94	1.21-7.17	0.021
	HPM 1371	4	24	55	23	0.07	0.02-0.22	0.000
Non-pathogenic group	*H0254*	1	42	10	53	0.13	0.02-1.06	0.026
	fimB	0	43	12	51	0	/	0.001
	vta1	9	34	28	35	0.33	0.14-0.80	0.014

VG +: *Number of isolates in the corresponding serovar but carrying the VG;* ***VG−:*** *Number of isolates in the corresponding serovar but no carrying the VG*
−VG +: *Number of isolates no in the corresponding serovar but carrying VG;* ***−VG −****: Number of isolates no in the corresponding serovar but no carrying VG*

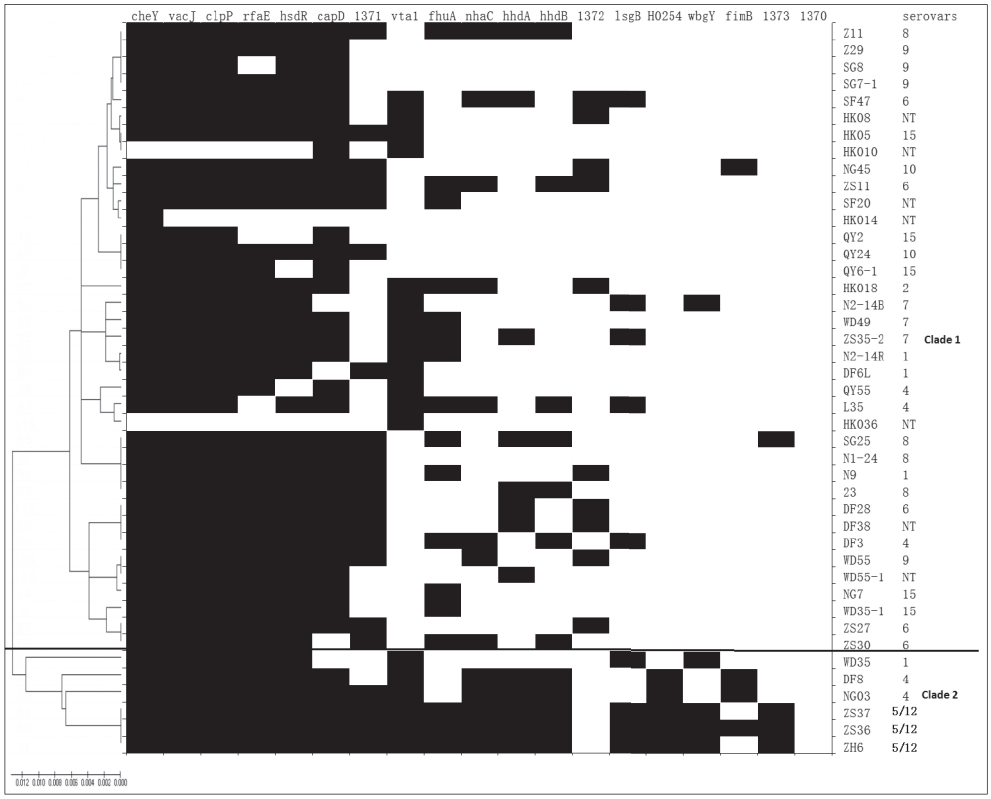


Fig 4. Neighbour-joining tree based on the MLST target sequences of 43 *G. parasuis* isolates

Oliveira and Pijoan [2] reported that *G. parasuis* was divided into three groups based on different serovars: highly pathogenic serovars (1, 5, 10, 12, 13, and 14), moderately pathogenic serovars (2, 4, and 15), and non-pathogenic serovars (3, 6, 7, 8, 9, and 11). The current study identified a significant correlation between different pathogenic serovar groups and several VGs. The highly pathogenic serovars had a significant positive association with *wbgY*, *fimB*, *1371*, and *1373*, and a significant negative association with *hhdB*. The moderately pathogenic serovars had a significant positive association with *hsdR* and *vta1,* and a significant negative association with *HPM1371*. The non-pathogenic serovars had a significant negative association with *H0254*, *fimB*, and *vta1* (P<0.05, *Table 3).*

Association Between ST and VGs

The ST analysis revealed two major clades (clade 1 and clade 2) based on the MLST target sequences of 43 *G. parasuis* isolates. Clade 1 includes 37 isolates of serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and NT, harboring 1 to 11 VGs each.

Table 4. Association between MLST clade and VGs of G. parasuis isolates

VG	Clade1+	Clade1−	Clade2+	Clade2−	OR	95% CI	P
vta1	13	24	6	0	0	/	0.004
nhaC	8	29	5	1	0.06	0.01-0.59	0.007
hhdA	8	29	5	1	0.06	0.01-0.59	0.007
hhdB	7	30	5	1	0.05	0.01-0.50	0.004
lsgB	5	32	4	2	0.08	0.01-0.56	0.01
H0254	0	37	5	1	0	/	0.000006
wbgY	1	36	4	2	0.01	0-0.14	0.001
fimB	1	36	3	3	0.03	0-0.38	0.006
HPM 1373	1	36	3	3	0.03	0-0.38	0.006

+: Number of isolates in the corresponding clade but carrying the VG; −: Number of isolates in the corresponding clade but no carrying the VG

Clade 2 includes 6 isolates of serovars 1, 4 and 5, harboring 8 to 16 VGs each *(Fig. 4)*. Interestingly, isolates in the second clade had a significantly increased probability of containing the VGs *vta1*, *nhaC*, *hhdA*, *hhdB*, *lsgB*, *H0254*, *wbgY*, *fimB*, and *1373* (P<0.05, *Table 4)*.

Discussion

In the study, a total of 117 *G. parasuis* isolates were obtained from 710 nasal swab samples from three provinces (Guangdong, Jiangxi, and Shanghai) in the south of China, the isolation rate was 16.5%, slightly higher than previous studies (14.6%) [26]. Ten distinct serovars were identified, serovars 10, 15, 6, and 8 were the dominant serovars identified in this study, with the detection frequency exceeding 10%. This differs from a previous report that the dominant serovars of strains in diseased pigs are 5 and 4 [27-31]. This difference may be uniquely associated with isolates from the nasal cavity of live piglets. In another study of *G. parasuis* isolates from the piglet nasal cavity by Zhang et al.[26], the dominant serovars in 6 provinces of China (Beijing, Shandong, Henan, Shanghai, Sichuan, and Chongqing) were 7, 3, 2, and 11 (over 10%). Those authors did not identify any isolates representing serovars 14 and 15. In the current study, we did not isolate any *G. parasuis* strains from serovars 3 and 11, and we only isolated a single strain from serovar 2. This suggests that serovars of *G. parasuis* from the swine nasal cavity exhibit a complex regional distribution across provinces in China. In both the current study and the study conducted by Zhang et al.[26], the detection frequency of serovars 4 and 5 was relatively low. Strains in serovars 4 and 5 are widely regarded as pathogenic strains, and they are most often identified from pigs with Glässer's disease. Although the detection frequency of serovars 4 and 5 was not high in live piglets, these isolates may nonetheless cause disease when an animal is under stress. Of note, the dominant serovars identified in this study, serovar 10 and serovar 15, were previously considered to be highly and moderately pathogenic, respectively. These two serovars have rarely been isolated in diseased pigs in China. Further attention and research are required to determine whether the presence of strains from serovars 10 and 15 in the respiratory tract of live piglets would cause localized disease, or even a potential disease epidemic.

In this study, all *G. parasuis* isolates were divided into four clusters according to the presence of VGs. Though serovars 2, 5, 8, 9, 10, and 15 were only distributed in one cluster, isolates belonging to the same serovar harbored different VGs. These differences were also present among strains that belonged to the same ST and serovar. For example, strains SG25 and N1-24, isolated from different farms, were both allocated to ST185 and serovar 8, and possessed seven identical VGs. However, strain SG25 had five more VGs than N1-24. Similarly, strains OY2 and QY6-1, isolated from the same farm, were allocated to ST255 and serovar 15, but strain QY6-1 has one more VG *(rfaE)* than OY2. Interestingly, strain QY6, isolated from the nasal cavity of the same piglet as strain QY6-1, also harbored *rfaE*. These results suggest that *G. parasuis* isolates may undergo multiple gene exchanges while coexisting in the respiratory tract. The VGs of isolates allocated to the same ST and serovar varied greatly, which may lead to differences in the pathogenicity and immunogenicity of strains belonging to the same ST and serovar. Once these strains invade the host tissues and organs, they may cause localized disease and eventually become epidemics. At that point, even if the serovars of commercially available vaccines and pathogenic strains were the same, the differences in VGs may lead to immune failures. That scenario would pose a substantial challenge to the development of a new vaccine.

Van et al.[31] reported that the detection frequency of the VGs *vta1*, *HPM-1371*, *capD*, *HPM-1372*, *lsgB*, *HPM-1373*, and *HPM-1370* was 62.5%, 35.7%, 30.3%, 12.5%, 8.9%, 8.9%, and 0%, respectively. Boerlin et al.[17] reported that the detection frequency of *vta1*, *hsdR*, *fimB*, *nhaC*, *fhuA*, *capD*, *wbgY*, and *H0254* was 92.5%, 47.9%, 37.2%, 38.3%,

38.3%, 23.4%, 22.3%, and 17%, respectively; Turni et al.[32] reported that the detection frequency of *hhdA* and *hhdB* was 36% and 13.3%, respectively, which differs from our results for most of the above VGs. Although previous studies [31] have shown that the VGs *lsgB*, *fhuA*, *capD*, *HPM-1372*, and *HPM-1373* were not observed in any isolates from non-pathogenic serovar group, our results showed that 8 of 43 isolates from the non-pathogenic serovar group were positive for *lsgB*, 16 were positive for *fhuA*, 39 were positive for *capD*, 8 were positive for *HPM-1372*, and 1 was positive for *HPM-1373*. Our results indicate that the distribution of VGs in *G. parasuis* is diverse and complex.

Olvera et al.[16] reported that isolates without *vtaA1* are generally avirulent. In this study, the presence of *vta1* was associated with a significantly decreased probability of membership in the non-pathogenic serovar group. This indicates that isolates allocated to the non-pathogenic serovar group may be avirulent based on this *vta1* analysis. Similarly, a significantly increased probability of harboring *vta1* was observed in the highly pathogenic serovars 1 and 5. Based on only the above analysis, the virulences predicted by the serovar and *vtaA1* analyses were consistent. However, all 21 serovar 10 isolates were *vtaA1* negative in the study, which indicates that serovar 10 isolates may be avirulent, but serovar 10 belonged to highly pathogenic serovars according to the previous research [2], so, the results of virulence prediction by the serovar and *vtaA1* analyses were in opposition. The correlation between serovars and VGs varied greatly among different serovars, even if the isolates belonged to the same pathogenic serovar group. For example, serovar 1 was only positively associated with *vta1*, while serovar 5 was positively associated with 9 VGs. Although the average number of VGs in the three pathogenic serovar groups was similar, the highly pathogenic serovars had a significant positive association with 4 VGs, the moderately pathogenic serovars had a significant positive association with 2 VGs, and no VGs had a positive association with non-pathogenic serovars. A previous study showed that *G. parasuis* MLST STs can be classified into two clades, with clade one almost completely containing avirulent or attenuated STs, and clade two mainly containing virulent STs [25,33]. In the current study, the detection frequency of VGs in clade two was much higher than that in clade one. While all isolates of clade two were *vtaA1* positive, only 30% of clade one isolates were *vtaA1* positive. We found a significant positive correlation between clade two and 9 VGs. Based on the VG analyses, it appears that isolates belonging to clade two are more virulent than isolates belonging to clade one. Overall, our results show that VG analyses may be a supplementary method for accurately allocating serovars or genotypes of *G. parasuis* into different pathogenic groups.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available upon request.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

LP and XYX conceived the experiments and wrote the paper. All authors performed the experiments. All authors have interpreted the data, revised the manuscript, and approved the final version.

References

1. Pilchová V, Seinige D, Hennig-Pauka I, Büttner K, Abdulmawjood A, Kehrenberg C: Development and validation of a loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Glaesserella (Haemophilus) parasuis*. *Microorganisms*, 9 (1): 41, 2020. DOI: 10.3390/microorganisms9010041

2. Oliveira S, Pijoan C: *Haemophilus parasuis*: New trends on diagnosis, epidemiology and control. *Vet Microbiol*, 99 (1): 1-12, 2004. DOI: 10.1016/j.vetmic.2003.12.001

3. Zhang Y, Dong Y, Xu Y, Wang Z, Yu N, Liu H, Wang L: The development of a SYBR green I multiple real-time fluorescence PCR assay for detection of *Actinobacillus pleuropneumoniae, Haemophilus parasuis* and *Pasteurella multocida*. *Kafkas Univ Vet Fak Derg*, 28 (1): 11-17, 2022. DOI: 10.9775/kvfd.2021.26202

4. Mahmmod YS, Correa-Fiz F, Aragon V: Variations in association of nasal microbiota with virulent and non-virulent strains of *Glaesserella (Haemophilus) parasuis* in weaning piglets. *Vet Res*, 51 (1): 7, 2020. DOI: 10.1186/s13567-020-0738-8

5. Blanco-Fuertes M, Correa-Fiz F, López-Serrano S, Sibila M, Aragon V: Sow vaccination against virulent *Glaesserella parasuis* shapes the nasal microbiota of their offspring. *Sci Rep*, 12 (1): 3357, 2022. DOI: 10.21203/rs.3.rs-1192355/v1

6. Kielstein P, Rapp-Gabrielson VJ: Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *J Clin Microbiol*, 30 (4): 862-865, 1992. DOI: 10.1128/jcm.30.4.862-865.1992

7. Peng L, Yuan X, Fang R, Liu-Fu W, Wen Q, Yang X: Serovars, antimicrobial susceptibility and molecular characteristics of *Haemophilus parasuis* isolates in Southern China. *Kafkas Univ Vet Fak Derg*, 26 (4): 491-497, 2020. DOI: 10.9775/kvfd.2019.23587

8. Hau SJ, Eberle KC, Brockmeier SL: Importance of strain selection in the generation of heterologous immunity to *Glaesserella (Haemophilus) parasuis*. *Vet Immunol Immunopathol*, 234:110205, 2021. DOI: 10.1016/j.vetimm.2021.110205

9. Dazzi CC, Guizzo JA, Prigol SR, Kreutz LC, Driemeier D, Chaudhuri S, Schryvers AB, Frandoloso R: New pathological lesions developed in pigs by a "non-virulent" strain of *Glaesserella parasuis*. *Front Vet Sci*, 7:98, 2020. DOI: 10.3389/fvets.2020.00098

10. Wan X, Li X, Osmundso T, Li C, Yan H: Whole-genome sequence analyses of *Glaesserella parasuis* isolates reveals extensive genomic variation and diverse antibiotic resistance determinants. *Peer J*, 8 (6):e9293, 2020. DOI: 10.7717/peerj.9293

11. Álvarez-Estrada A, Gutiérrez-Martín CB, Rodríguez-Ferri EF, Martínez-Martínez S: Transcriptomics of *Haemophilus (Glasserella) parasuis* serovar 5 subjected to culture conditions partially mimetic to natural infection for the search of new vaccine antigens. *BMC Vet Res*, 14 (1): 326, 2018. DOI: 10.1186/s12917-018-1647-1

12. Angen O, Oliveira S, Ahrens P, Svensmark B, Leser TD: Development of an improved species specific PCR test for detection of *Haemophilus parasuis*. *Vet Microbiol*, 119 (2-4): 266-276, 2007. DOI: 10.1016/j.vetmic.2006.10.008

13. Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW, BRaDP1T Consortium: Development of a multiplex PCR assay for rapid molecular serotyping of *Haemophilus parasuis*. *J Clin Microbiol*, 53 (12): 3812-3821, 2015. DOI: 10.1128/JCM.01991-15

14. Sack M, Baltes N: Identification of novel potential virulence-associated factors in *Haemophilus parasuis*. *Vet Microbiol*, 136 (3-4): 382-386, 2009. DOI: 10.1016/j.vetmic.2008.11.008

15. Zhou H, Yang B, Xu F, Chen X, Wang J, Blackall PJ, Zhang P, Xia Y, Zhang J, Ma R: Identification of putative virulence-associated genes of *Haemophilus parasuis* through suppression subtractive hybridization. *Vet Microbiol*, 144 (3-4): 377-383, 2010. DOI: 10.1016/j.vetmic.2010.01.023

16. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters *(vtaA). Vet J*, 191 (2): 213-218, 2012. DOI: 10.1016/j.tvjl.2010.12.014

17. Boerlin P, Poljak Z, Gallant J, Chalmers G, Nicholson V, Soltes GA, MacInnes JI: Genetic diversity of *Haemophilus parasuis* from sick and healthy pigs. *Vet Microbiol*, 167 (3-4): 459-467, 2013. DOI: 10.1016/j.vetmic.2013.07.028

18. Wang X, Xu X, Wu Y, Li L, Cao R, Cai X, Chen H: Polysaccharide biosynthesis protein capD is a novel pathogenicity-associated determinant of *Haemophilus parasuis* involved in serum-resistance ability. *Vet Microbiol*, 164 (1-2): 184-189, 2013. DOI: 10.1016/j.vetmic.2013.01.037

19. Zhang B, Yu Y, Zeng Z, Ren Y, Yue H: Deletion of the *rfaE* gene in *Haemophilus parasuis* sc096 strain attenuates serum resistance, adhesion and invasion. *Microb Pathog*, 74, 33-37, 2014. DOI: 10.1016/j.micpath.2014.07.006

20. Lawrence P, Bey R: Map-based comparative genomic analysis of virulent *Haemophilus parasuis* serovars 4 and 5. *J Genomics*, 3, 59-71, 2015. DOI: 10.7150/jgen.10924

21. He L, Wen X, Yan X, Ding L, Cao S, Huang X, Wu R, Wen Y: Effect of *cheY* deletion on growth and colonization in a *Haemophilus parasuis* serovar 13 clinical strain EP3. *Gene,* 577 (1): 96-100, 2016. DOI: 10.1016/j.gene.2015.11.046

22. Huang J, Wang X, Cao Q, Feng F, Xu X, Cai X: *ClpP* participates in stress tolerance and negatively regulates biofilm formation in *Haemophilus parasuis*. *Vet Microbiol*, 182, 141-149, 2016. DOI: 10.1016/j.vetmic.2015.11.020

23. Zhao L, Gao X, Liu C, Lv X, Jiang N, Zheng S: Deletion of the *vacJ* gene affects the biology and virulence in *Haemophilus parasuis* serovar 5. *Gene*, 603, :42-53, 2017. DOI: 10.1016/j.gene.2016.12.009

24. Olvera A, Cerda-Cuellar M, Aragon V: Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing. *Microbiolgy*, 152 (12): 3683-3690, 2006. DOI: 10.1099/mic.0.29254-0

25. Mullins MA, Register KB, Brunelle BW, Aragon V, Galofré-Mila N, Bayles DO, Jolley KA: A curated public database for multilocus sequence typing (MLST) and analysis of *Haemophilus parasuis* based on an optimized typing scheme. *Vet Microbiol*, 162 (2-4): 899-906, 2013. DOI: 10.1016/j.vetmic.2012.11.019

26. Zhang P, Zhang C, Aragon V, Zhou X, Zou M, Wu C, Shen Z: Investigation of *Haemophilus parasuis* from healthy pigs in China. *Vet Microbiol*, 231, 40-44, 2019. DOI: 10.1016/j.vetmic.2019.02.034

27. Qi B, Li F, Chen K, Ding W, Xue Y, Wang Y, Wang H, Ding K, Zhao Z: Comparison of the *Glaesserella parasuis* virulence in mice and piglets. *Front Vet Sci*, 8:659244, 2021. DOI: 10.3389/fvets.2021.659244

28. Macedo N, Gottschalk M, Strutzberg-Minder K, Van CN, Zhang L, Zou G, Zhou R, Marostica T, Clavijo MJ, Tucker A, Aragon V: Molecular characterization of *Glaesserella parasuis* strains isolated from North America, Europe and Asia by serotyping PCR and LS-PCR. *Vet Res,* 52 (1): 68, 2021. DOI: 10.1186/s13567-021-00935-9

29. Wang Z, Zhao Q, Wei H, Wen X, Cao S, Huang X, Wu R, Yan Q, Huang Y, Wen Y: Prevalence and seroepidemiology of *Haemophilus parasuis* in Sichuan province, China. *Peer J*, 5:e3379, 2017. DOI: 10.7717/peerj.3379

30. Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J, Liu Y: Characterization of Chinese *Haemophilus parasuis* isolates by traditional serotyping and molecular serotyping methods. *Plos One*, 11 (12):e0168903, 2016. DOI: 10.1371/journal.pone.0168903

31. Van CN, Thanh TVT, Zou G, Jia M, Wang Q, Zhang L, Ding W, Huang Q, Zhou R: Characterization of serotypes and virulence genes of *Haemophilus parasuis* isolates from Central Vietnam. *Vet Microbiol*, 230 (1): 117-122, 2019. DOI: 10.1016/j.vetmic.2019.02.008

32. Turni C, Singh R, Blackall PJ: Virulence-associated gene profiling, DNA fingerprinting and multilocus sequence typing of *Haemophilus parasuis* isolates in Australia. *Aust Vet J*, 96 (6): 196-202, 2018. DOI: 10.1111/avj.12705

33. Wang L, Ma L, Liu Y, Gao P, Li Y, Li X, Liu Y: Multilocus sequence typing and virulence analysis of *Haemophilus parasuis* strains isolated in five provinces of China. *Infect Genet Evol*, 44, 228-233, 2016. DOI: 10.1016/j.meegid.2016.07.015