Serovar and Virulence Genes of Glaesserella (Haemophilus) Parasuis Isolates from the Nasal Cavity of Live Piglets

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Abstract

Glaesserella (Haemophilus) parasuis (G. parasuis) is a commensal bacterium in the swine upper respiratory tract that can cause Glässer's disease, particularly in piglets. In this study, we detected the serovars and 19 known virulence genes (VGs) of H. parasuis isolates from the nasal cavities of live piglets from the south of China. Serovar 10 (17.9%) was the most prevalent, followed by serovars 15 (14.5%), 6 (12.0%), 8 (11.1%), 4 (8.5%), 7 (7.7%), 9 (7.7%), 1 (7%), 5/12 (4.3%), and 2 (0.9%). This differs from previous studies on common G. parasuis serovars. The detection rate of 19 VGs ranged from 1.7% to 95.2%, with vacJ and clpP (95.7%) as the most prevalent. The G. parasuis isolates belonging to the same sequence type 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 MLST). To our knowledge, this is the first research to examine the characteristics of G. parasuis nasal cavity isolates from live piglets in the south of China. 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.

Introduction

Glaesserella (Haemophilus) parasuis (G. parasuis), the pathogen that causes Glässer's disease, has brought huge economic losses to the global swine industry (Bouchet et al., 2008; Oliveira and Pijoan, 2004). 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 (Cerdà-Cuéllar et al., 2010; Galofré-Milà et al., 2017; Maclnnes et al., 2008).

To date, 15 serovars have been identified, in addition to some non-typable (NT) strains (Kielstein P and Rapp-Gabrielson, 1992; Jin et al., 2006). Serovar identification of the isolates is the main basis for designing vaccination programs (Liu et al., 2016). Some earlier studies suggested that G. parasuis serovars were a virulence marker and could be divided into three pathogenic groups (Oliveira and Pijoan, 2004). 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 (Angen et al., 2004; Aragon et al., 2010; Boerlin et al., 2013; Zhang et al., 2019). Thus, it remains unclear whether serovar can be used as a marker of virulence in G. parasuis.

Comparing the pathogenicity difference between wild-type strains and gene knockout mutants is an important method for evaluating the role of putative VGs in pathogenicity. In addition, molecular epidemiology and bioinformatics studies are often used to discover and evaluate putative VGs, and some VGs have already been evaluated by these methods. It is generally believed that a single 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 three provinces in the south of China. Our results provide more information on the epidemiology and pathogenesis of *G. parasuis*.

**Materials And Methods**

**Identification and serotyping**

One hundred seventeen *G. parasuis* isolates were isolated 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. The *G. parasuis* strains were identified by NAD-dependency and 16S rRNA PCR (Angen et al., 2007). The isolates underwent molecular serotyping via a multiplex PCR assay described in Howell et al. (2015).

**VG analysis**

Nineteen VGs were analyzed using PCR as previously described—details of all primers used are listed in Table 1.

**MLST**

A MLST analysis was carried out using a method previously described (Olvera et al., 2006; Mullins et al., 2013). A neighbor-joining tree was built using the MEGA version 5.0 software based on the MLST target sequences.

**Table 1** Primers used to amplify VGs
| VGs | Primers | Sequence (5’ → 3’) | Product size | References |
|-----|---------|---------------------|--------------|------------|
| hhdA | hhdAF | GGTTCTAGTTCACAACACAGCCAATAAC | 964 | Sack et al., 2009 |
|     | hhdAR | GATATTACCCTGCTTCTATTGTAC | | |
| hhdB | hhdBF | ATCTTGCCCTGATTAGAGAGTGGAGT | 557 | |
|     | hhdBR | GTGAAATATAGCCCCTATCCAAATAGG | | |
| fhuA | fhuAF | ATGGTTGGTTGTAATGGGAT | 563 | Zhou et al., 2010 |
|     | fhuAR | AACAAACCCGAGCTAGGTGAT | | |
| vta1 | vta1F | TTTAGGTAAGATAAGCAAGGAATCC | 406 | Olvera et al., 2012 |
|     | vta1R | CCACAGAAAAACCTACCCCTCCT | | |
| wbgY | wbgYF | TTAGGGCTTGTCGCCCTATTTC | 380 | Boerlin et al., 2013 |
|     | wbgYR | GAAGCAGATATGTAATACCACG | | |
| fimB | fimBF | CTAAGAGAGAGCAGGCGATAGA | 386 | |
|     | fimBR | TGGTACACACAATGGCTACGTTGA | | |
| hsdR | hsdRF | GCAAGCTTACTCTCGTACTAACC | 410 | |
|     | hsdRR | AGGCTCCACATAGTTCTCTACT | | |
| nhaC | nhaCF | CATATTGTGGTTACAAGGTTGGCGAG | 415 | |
|     | nhaCR | CTAATACGGAAGTCACTGTACCACG | | |
| H0254 | H0254F | CAGTGAAAGTCTGTGATTGGAACC | 397 | |
|     | H0254R | GAGCGTTCGTTACATCTTTGTT | | |
| capD | capDF | CGAAGGGAGTGGTCTATCA | 958 | Wang et al., 2013 |
|     | capDR | GAGTTTCTCACCAGGTCTAA | | |
| rfaE | rfaEF | GCAGGGCGAGGCTGGTAA | 524 | Zhang et al., 2014 |
|     | rfaER | TGGGTCGGTGAAATGGAATGG | | |
| lsgB | lsgBF | ATGAATTTTGTATTTTGTATGACTCCATT | 969 | Lawrence et al., 2015 |
|     | lsgBR | CTATTGGCAGTGTAGTCAATTTCT | | |
| HPM1370 | HPM1370F | ATGCTAAAAAGAGTGTGGATATTTC | 540 | |
|     | HPM1370R | TATATTGATTAACATAATC | | |
| HPM1371 | HPM1371F | ATGAACTTTCTACCATTGCGCCCTTCCC | 520 | |
|     | HPM1371R | ATTATTTGAAATCCAGGTCTATG | | |
Statistical analyses

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

Results

Identification and serotyping

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. 1A). 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. 1B).

VG analysis of the G. parasuis isolates

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, 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 positive correlation was found between the following: serovar 1 and vta1; serovar 4 and hhdB, H0254, nhaC, and vta1; serovar 5 and fhuA, wbgY, hhdB, hhdA, 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 rfaE, 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      | 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|
Oliveira and Pijoan (2004) 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).

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

|   | VG   | Number of isolates in the corresponding serovar but carrying the 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.
|---|---|---|---|---|---|
| HPM 1371 | 2 | 15 | 60 | 40 | 0.09 | 0.02-0.42 | 0.0000336 |
| 8 | hhdA | 9 | 4 | 30 | 74 | 5.55 | 1.59-19.41 | 0.009 |
| 8 | hhdB | 5 | 5 | 23 | 81 | 5.63 | 1.68-18.87 | 0.005 |
| 0 | 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 |
| 7 | 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 | 0.0000289 |
| NT | vacJ | 6 | 5 | 106 | 0 | 0 | / | 0.000003 |
| NT | cheY | 7 | 4 | 102 | 4 | 0.07 | 0.01-0.34 | 0.003 |
| NT | clpP | 7 | 4 | 105 | 1 | 0.02 | 0-0.20 | 0.000212 |
| NT | 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.

|                  | VGs  | VG + | VG-  | −VG + | −VG-  | OR   | 95% CI     | P    |
|------------------|------|------|------|-------|-------|------|------------|------|
| high pathogenic  | wbgY | 7    | 28   | 1     | 70    | 17.5 | 2.06-148.84| 0.002|
| group           |      |      |      |       |       |      |            |      |
|                  | 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|
|                  | HPM1371 | 27 | 8    | 32    | 39    | 4.11 | 1.64-10.28 | 0.002|
|                  | HPM1373 | 6  | 29   | 1     | 70    | 14.48| 1.67-125.66| 0.005|
| moderately       | hsdR | 22   | 6    | 78    | 0     | 0    | /          | 0.0002|
| pathogenic       |      |      |      |       |       |      |            |      |
| group           | vta1 | 15   | 13   | 22    | 56    | 2.94 | 1.21-7.17  | 0.021|
|                  | HPM1371 | 4  | 24   | 55    | 23    | 0.07 | 0.02-0.22  | 0.000|
| non-pathogenic   | H0254 | 1    | 42   | 10    | 53    | 0.13 | 0.02-1.06  | 0.026|
| group           |      |      |      |       |       |      |            |      |
|                  | fimB | 0    | 43   | 12    | 51    | 0    | /          | 0.001|
|                  | vta1 | 9    | 34   | 28    | 35    | 0.33 | 0.14-0.80  | 0.014|

**Association between MLST and VGs**

The MLST 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. 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).

**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

**Discussion**

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 (Cai et al., 2005; Castilla et al., 2012; Van et al., 2019; Ma et al., 2016; Zhou et al., 2010). 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., 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. (2019), the detection frequency of serovars 4 and 5/12 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 5/12 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. (2019) 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. (2013) reported that the detection frequency of *vta1*, *hsdR*, *mB*, *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. (2018) 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 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. (2012) 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 highly pathogenic isolates from serovar 10 were *vtaA1* negative, which indicates that the highly pathogenic serovar 10 isolates may be avirulent. Thus, 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 (Mullins et al., 2013; Wang et al., 2017). In the current study, the detection frequency of VGs in clade two was much higher than that in clade one. While all clade two isolates 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.

**Conclusion**

We analyzed the serovars and the VGs of 117 *G. parasuis* isolates from the nasal cavity of live piglets in the south of China. The distribution of serovars and VGs was distinct from previous studies, and we discovered considerable genetic heterogeneity among nasal isolates. Our results complement epidemiological data of *G. parasuis* and aid our understanding of the extreme genetic diversity associated with this important bacterial species.

**Declarations**

**Authors Contributions** All authors contributed to the study conception and design. Ling Peng conceived the experiments and wrote the paper. All authors performed the experiments. All authors read and approved the final manuscript.

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**Conflicts of interest** The authors declare that they have no conflict of interest.

**Data availability** All data generated and analysed during this study are included in this published article.

**Consent to participate** Not applicable.

**Ethics approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Consent for publication** All authors give consent for publication.

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**References**
1. 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

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

3. Aragon V, Cerdà-Cuéllar M, Fraile L, Mombarg M, Nofrarías M, Olvera A, Sibila M, Solanes D, Segalés J (2010). Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains. Vet Microbiol 142:387–393. https://doi.org/ 10.1016/j.vetmic.2009.10.025

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

5. Bouchet B, Vanier G, Jacques M, Gottschalk M (2008). Interactions of *Haemophilus parasuis* and its LOS with porcine brain microvascular endothelial cells. Vet Res 39:42. https://doi.org/ 10.1051/vetres:2008019

6. Cai X, Chen H, Blackall P, 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

7. Castilla KS, de Gobbi DDS, Moreno LZ, Paixã 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

8. Cerdà-Cuéllar M, Naranjo JF, Verge A, Nofrarías M, Cortey M, Olvera A, Segalés J, Aragon V (2010). Sow vaccination modulates the colonization of piglets by *Haemophilus parasuis*. Vet Microbiol 145:315-320. https://doi.org/ 10.1016/j.vetmic.2010.04.002

9. Galofré-Milà N, Correa-Fiz F, Lacouture S, Gottschalk M, Strutzberg-Minder K, Bensaid A, Pina-Pedrero S, Aragon V (2017). A robust PCR for the differentiation of potential virulent strains of *Haemophilus parasuis*. BMC Vet Res 13: 124. https://doi.org/ 10.1186/s12917-017-1041-4

10. He LQ, Wen XT, Yan XF, Ding LQ, Cao SJ, Huang XB, Wu R, Wen YP (2016). Effect of cheY deletion on growth and colonization in a *Haemophilus parasuis* serovar 13 clinical strain EP3. Gene 577: 96-100. https://doi.org/ 10.1016/j.gene.2015.11.046

11. Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø, Aragon V, Williamson SM (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

12. Huang JV, Wang XR, Cao Q, Feng FF, Xu XJ, Cai XW (2016). ClpP participates in stress tolerance and negatively regulates biofilm formation in *Haemophilus parasuis*. Vet Microbiol 182:141-149. https://doi.org/ 10.1016/j.vetmic.2015.11.020

13. Jin H, Zhou R, Kang MS, Luo R, Xuwang Cai XW (2006). Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*. Vet Microbiol 118:117-123.
14. 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. https://doi.org/10.1016/0168-1656(92)90094-P

15. Lawrence P, Bey R 2015 Map-based comparative genomic analysis of virulent *Haemophilus parasuis* serovars 4 and 5. J Gen 3:59–71. https://doi.org/10.7150/jgen.10924

16. Liu HS, Xue Q, Zeng QY, Zhao ZQ 2016 *Haemophilus parasuis* vaccines. Vet Immunol Immunop 180:53-58. https://doi.org/10.1016/j.vetimm.2016.09.002

17. MacInnes JI, Gottschalk M, Lone AG, Metcalf DS, Ojha S, Rosenda T, Watson SB, Friendship RM 2008 Prevalence of *Actinobacillus pleuropneumoniae, Actinobacillus suis, Haemophilus parasuis, Pasteurella multocida,* and *Streptococcus suis* in representative Ontario swine herds. Can J Vet Res 72:242–248. https://doi.org/10.1111/j.1751-0813.2008.00260.x

18. Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J 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

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

20. 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

21. Olvera A, Cerda-Cuellar M, Aragon V 2006 Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing. Vet Microbiol 152:3683–3690. https://doi.org/10.1016/j.vetmic.2012.11.019

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

23. Sack M, Baltes N 2009 Identification of novel potential virulence-associated factors in *Haemophilus parasuis*. Vet Microbiol 136:382–386. https://doi.org/10.1016/j.vetmic.2008.11.008

24. Turni C, Singh R, Blackall PJ 2018 Virulence-associated gene profiling, DNA fingerprinting and multilocus sequence typing of *Haemophilus parasuis* isolates in Australia. Aust Vet J 96:96-202. https://doi.org/10.1111/avj.12705

25. Wang LY, Ma LN, Liu YG, Gao PC, Li YQ, Li XR, Liu YS. Multilocus sequence typing and virulence analysis of *Haemophilus parasuis* strains isolated in five provinces of China. Infect Genet Evol 44:228–233. https://doi.org/10.1016/j.meegid.2016.07.015

26. Wang X, Xu X, Wu Y, Li L, Cao R, Cai X, Chen HC 2013 Polysaccharide biosynthesis protein capd is a novel pathogenicity-associated determinant of *Haemophilus parasuis* involved in serum-resistance ability. Vet Microbiol 164:184-189. https://doi.org/10.1016/j.vetmic.2013.01.037
27. Van CN, Thanh TVT, Zou G, Jia M, Wang QN, Zhang LJ, Ding WG, Huang Q, Zhou R 2019 Characterization of serotypes and virulence genes of *Haemophilus parasuis* isolates from Central Vietnam. Vet Microbiol 230:117-122. https://doi.org/10.1016/j.vetmic.2019.02.008

28. Zhang B, Yu Y, Zeng Z, Ren Y, Yue H 2014 Deletion of the rfae gene in *Haemophilus parasuis* sc096 strain attenuates serum resistance, adhesion and invasion. Microb Pathogenesis 74:33-37. https://doi.org/10.1016/j.micpath.2014.07.006

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

30. Zhang P, Zhang CY, Aragon V, Zhou X, Zou M, Wu CM, Shen ZQ 2019 Investigation of *Haemophilus parasuis* from healthy pigs in China. Vet Microbiol 231:40-44. https://doi.org/10.1016/j.vetmic.2019.02.034

31. Zhou HZ, Yang B, Xu FZ, Chen XL, Wang JL, Blackall PJ, Zhang PJ, Xia YH, Zhang J, Ma RC 2010 Identification of putative virulence-associated genes of *Haemophilus parasuis* through suppression subtractive hybridization. Vet Microbiol 144:377-383. https://doi.org/10.1016/j.vetmic.2010.01.023

32. Zhou XL, Xu XJ, Zhao YX, Chen P, Zhang X, Cai XW 2010 Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates. Vet Microbiol 141:168–173. https://doi.org/10.1016/j.vetmic.2009.05.012

**Figures**

![Figure 1](image)

**Figure 1**

The distribution of serovar in all 117 isolates (A) and in different provinces (B)
Figure 2

The distribution of 19 VGs in all 117 isolates
Figure 3

Clustering of G. parasuis isolates based on VGs
Figure 4

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