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Research Article

Serovars, Antimicrobial Susceptibility and Molecular Characteristics of *Haemophilus parasuis* Isolates in Southern China

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Abstract

This study analyzed the characteristics of 133 *Haemophilus parasuis* isolates in southern China. These isolates belonged to eleven serovars (1, 2, 4-10, 13 and 15) with 12.0% of them being characterised as non-typable. A relatively high level in resistance was encountered for trimethoprim + sulfamethoxazole (89.9%), tetracycline (75.3%), amoxicillin (69.1%), streptomycin (63.6%), carbenicillin (60.2%), kanamycin (46.6%) and ampicillin (45.6%). A total of 60% of the isolates were negative for group 1 virulence-associated autotransporters *(vtaA)*. All group 1 *vtaA* negative isolates fell into polyacrylamide gel electrophoresis (PAGE) type I, while all group 1 *vtaA* positive isolates were classified as PAGE type II. The results of Multi-locus sequence typing (MLST) indicated a high degree of variation, 45 isolates in the study were assigned into 31 sequence types with 28 of these being new (not found in the MLST database). Antimicrobial resistance was observed in every serovar, there was no statistically significant correlation between the antimicrobial resistance and the serovars. The isolates allocated to clade 2 (based on MLST target sequences) showed the molecular characteristics of highly pathogenic strains in whole-cell protein profiling, *vtaA* groups 1, superoxide dismutase *(sodA)* sequence and MLST.

Keywords: *Haemophilus parasuis, Antimicrobial susceptibility, MLST, PAGE, vtaA, SodA*

Güney Çin'de Haemophilus parasuis İzolatlarının Serovarları, Antimikrobiyal Duyarlılıkları ve Moleküler Özellikleri

Öz

Bu çalışmada, Güney Çin'deki 133 H. parasuis izolatının özellikleri analiz edildi. Bu izolatlar, %12.0'ı tiplendirilemeyen olarak karakterize edilen on bir serovara (1, 2, 4-10, 13 ve 15) aitti. Trimetoprim + sülfametoksazol (%89.9), tetrasiklin (%75.3), amoksisilin (%69.1), streptomisin (%63.6), karbenisilin (%60.2), kanamisin (%46.6) ve ampisilin (%45.6) için nispeten yüksek bir direnç seviyesine rastlandı. İzolatların %60'ı, grup 1 virülansa bağlı ototransportörler (vtaA) yönünden negatifti. Tüm grup 1 vtaA negatif izolatları poliakrilamid jel elektroforezi (PAGE) tip I'e dahil edilirken, tüm grup 1 vtaA pozitif izolatları PAGE tip II olarak sınıflandırıldı. Multi-lokus sekans tiplendirmesinin (MLST) sonuçları yüksek derecede bir varyasyon gösterdi. Çalışmadaki 45 izolat 31 sekans tipine atanırken bunların 28'inin yeni olduğu belirlendi (MLST veritabanında bulunamadı). Her serovarda antimikrobiyal direnç gözlendi, antimikrobiyal direnç ile serovarlar arasında istatistiksel olarak anlamlı bir ilişki yoktu. Clade 2'ye ayrılan izolatlar (MLST hedef sekanslarına dayanarak) hücre proteini profillemesi, vtaA grupları 1, süperoksit dismutaz (sodA) dizilimi ve MLST sonuçları patojenitesi yüksek suşların moleküler özelliklerini gösterdi.

Anahtar sözcükler: *Haemophilus parasuis, Antimikrobiyal duyarlılık, MLST, PAGE, vtaA, SodA*

INTRODUCTION

Haemophilus parasuis (H. parasuis) is a Gram-negative bacterium that colonizes the upper respiratory tract of pigs. After invasion of the host, it causes Glasser's disease, which is associated with fibrinous polyserositis, meningitis, and arthritis. The infection of *H. parasuis* can be controlled by the use of serovar specific vaccines and antibiotics [1-3].

A total of 15 serovars and a large number of non-typable (NT) strains have been identified [3]. The high level of intrinsic diversity among *H. parasuis* populations has hindered

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the development of effective cross-protective vaccines [3]. Although antibiotic treatment is the most common intervention in the control of Glasser's disease, the use of antibiotics may lead to increased antibiotic resistance [4], therefore, antimicrobial susceptibility testing is a crucial step prior to antibiotic prescription.

Multi-locus sequence typing (MLST) discriminates among isolates by comparing DNA sequences of six to ten house-keeping genes [5]. As an objective and highly standardized method, the MLST allows full characterization of all sampled isolates. In *H. parasuis* research, Olvera et al.[6] were the first to use the MLST method to genetically characterize *H. parasuis* isolates. Mullins et al.[7] then optimized the MLST-based analysis of population structure and genetic diversity among *H. parasuis* populations.

H. parasuis isolates range from highly virulent to non-pathogenic. Although the molecular basis underlying the virulence of many *H. parasuis* isolates has not been completely established, some virulence markers or factors have been proposed [7-11]. In this study, the antimicrobial susceptibility of 133 isolates of *H. parasuis* from southern China was tested against 26 antimicrobial agents. These isolates were characterized by serotyping, whole-cell protein profiling, identification of virulence-associated autotransporters *(vtaA),* and the sequencing of the superoxide dismutase *(sodA)* gene and by MLST. A phylogenetic analysis between the 45 isolates based on MLST target sequences was also conducted.

MATERIAL and METHODS

Serotyping of H. parasuis Isolates

A total of 133 *H. parasuis* isolates were collected either from the blood, joint fluid, lungs and nasal cavity of possibly diseased pigs accompanied by symptoms associated with Glässer's disease or from the nasal cavity of healthy pigs between 2007 and 2016 in southern China; A total of 88% of the 133 isolates were isolated from the nasal cavity of pigs, and 12% of the isolates were from blood, joint fluid, and lungs of pigs. Identification of the isolates was carried out by nicotinamide adenine dinucleotide (NAD) dependency, biochemical tests and using PCR [12]. The isolates were serotyped using a multiplex PCR method [13].

Antimicrobial Susceptibility

The antimicrobial susceptibility of the *H. parasuis* isolates was assessed by the disk diffusion method with the use of blood agar medium with 0.0025% of NAD [14]. Antimicrobial disks (Hangzhou Tianhe Microbiological Co., Hangzhou, China) and associated concentrations are described in *Table 1*. Growth inhibition was assessed against Clinical and Laboratory Standards Institute standards and the isolates were classified as susceptible, intermediate or resistant [15,16]. *Actinobacillus pleuropneumoniae* (ATCC 27090) and *Escherichia coli* (ATCC 25922) reference strains were used as experimental controls.

Whole-cell Protein Profiling and Identification of Virulence-associated Autotransporters

The whole-cell protein profiles of *H. parasuis* isolates were evaluated using the method described by Oliveira *and* Pijoan [9]. And the polyacrylamide gel electrophoresis (PAGE) types were identified. Isolates containing major proteins weighing between 36 and 38 kDa, were classified as PAGE type II and isolates lacking this group of proteins were classified as PAGE type I. All *H. parasuis* isolates were grown under the same culture conditions. The *vtaA* of the *H. parasuis* isolates were identified using the method described in Olvera et al.[10]. Accordingly, a multiplex PCR was used for the diagnosis of *H. parasuis* at the species level (group 3 *vtaA* positive) and to differentiate putative non-virulent isolates (group 1 *vtaA* negative).

Table 1. *Antimicrobial resistance profiles of H. parasuis isolates from southern China*

Antibiotic Tested (µg)	Level of Susceptibility		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Erythromycin (15)	59.6	37.5	2.9
Tilmicosin (15)	100	0	0
Amoxicillin (10)	29.1	1.8	69.1
Ampicillin (10)	33.8	20.6	45.6
Carbenicillin (100)	30.5	9.3	60.2
Cefazolin (30)	91.3	2.5	6.3
Cefalexin (30)	80.4	8.4	11.2
Cefuroxime (30)	84.9	0.8	14.3
Ceftiofur (30)	100	0	0
Ceftriaxone (30)	100	0	0
Cefalotin (30)	100	0	0
Gentamicin (10)	79.6	4.9	15.4
Kanamycin (30)	41.0	12.4	46.6
Amikacin (30)	59.1	15.2	25.6
Spectinomycin (100)	95.1	0.0	4.9
Streptomycin (10)	22.7	13.6	63.6
Tetracycline (30)	17.8	6.8	75.3
Deoxytetracycline (30)	100	0	0
Enrofloxacin (5)	78.2	21.0	0.8
Ciprofloxacin (5)	66.5	26.1	7.5
Norfloxacin (10)	80.9	11.1	8.0
Ofloxacin (5)	100	0	0
Trimethoprim (1.25) + Sulfamethoxazole (23.75)	8.2	1.9	89.9
Lincomycin (2)	29.4	43.7	26.9
Rifampicin (5)	100	0	0
Albamycin (5)	100	0	0

MLST

A total of 45 isolates were selected for the MLST analysis and 7 housekeeping genes were used in the MLST analysis of the *H. parasuis* isolates: malate dehydrogenase gene *(mdh),* β chain of ATP synthase *(atpD),* translation initiation factor IF-2 *(infB),* ribosomal protein β subunit *(rpoB),* 6-phosphogluconate dehydrogenase(6pgd), glyceraldehyde -3-phosphate dehydrogenase *(g3pd)* and fumarate reductase B *(frdB)*. These genes were amplified with primers described in Mullins et al.[7]. The PCR reaction conditions were: 5 min at 95°C; 35 cycles of 95°C for 1 min, 48°C for 30 s and 72°C for 30 s, followed by a final elongation step of 72°C for 10 min. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China), using the dideoxy chain-termination method. The housekeeping gene sequences, obtained for each isolate, were edited, assembled, and aligned by the software MEGA version 5.0 [17]. Then, the sequences were submitted to the MLST database *(http://pubmlst.org/hparasuis)* for the analysis of allele number and sequence type (ST) according to the methodology described by Jolley *and* Maiden [18]. A Neighbor-Joining tree, based on the Maximum Composite Likelihood distance estimation model with 1000 bootstrap replicates, was created from the concatenated sequences of the seven housekeeping genes [7].

Characterizing the Genetic Diversity of sodA

Le [19] and Chen et al.[20] reported there are some changs in *sodA* amino acid sites between virulent and avirulent strains of *H. parasuis*, focusing on changes in four *sodA* amino acid sites (at the 16th position: Asp-to-Glu, 49th position: Leu-to-Phe, 69th position: Arg-to-Gln, and 186th position Ile-to-Val). In the current study, the *sodA* gene of the 45 isolates were PCR amplified using the primers *sodA* F (5'ATG-GCATACACAT TACCTGAG T TAGA3') and *sodA* R (5'TTATG-CTTGGGAT TCAAAACGT3'). Subsequently, the PCR product obtained from each isolate was sub-cloned into the pUCm-T vector and sequenced using the dideoxy chain termination method (Sangon Biotech Ltd., Shanghai, China).

Statistical Analysis

A neighbour-joining tree derived from the MLST target sequences of 45 *H. parasuis* isolates was constructed by using MEGA version 5.0 software. An x^2-test was used to assess correlation between the antimicrobial resistance and the serovars, and a resulting P value lower than 0.05 was regarded as significant.

RESULTS

From the 133 serotyped *H. parasuis* isolates, eleven distinct serovars were identified. Serovar 10 (15.8%) was the most prevalent, followed by serovars 15 (12.8%), 6 (12.0%), 8 (9.8%), 5 (8.3%), 4 (7.6%), 9 (7.5%), 1 (6.8%), 7 (5.3%), 13 (1.5%) and 2 (0.8%). A total of 12.0% of the isolates tested were NT *(Table 2)*.

We tested the antibiotic resistance of the 133 isolates of *H. parasuis* against 26 different antibiotics in this study. The resistance rates to 7 antibiotics were over 40%, to 5 antibiotics were 10%-30% and to 6 antibiotics were below 10%, while, the resistance rates to 8 antibiotics were 0% *(Table 1)*. The resistance to 26 antimicrobial agents among all these isolates was counted and compared it with the distribution of serovars to the distribution of antimicrobial resistance. We found that the isolation frequency in serovar 5, 1, 7 and 8 was a little higher than the incidence of antimicrobial resistance in the corresponding serovar, while, the isolation frequency in serovar 6 and 9 was a little lower than the incidence of antimicrobial resistance in the corresponding serovar, but there was not significant difference (x^2-test, P>0.05). For other serovars, the isolation frequency of each serovar was very similar to the incidence of antimicrobial resistance in the corresponding serovar *(Fig. 1)*.

A total of 39.8% of the isolates were classified as PAGE type II, including some of those included in serovars 1, 2, 4, 5, 6, 7, 9, 13, 15 and some NT isolates *(Table 2)*. A total of 60.2% of the isolates were classified as PAGE type I, including some of those included in serovars 1, 4, 6, 8, 9, 10, 15, and some NT isolates *(Table 2)*.

In this study, all isolates were positive for group 3 *vtaA* and 60% of the isolates were negative for group 1 *vtaA*. All group 1 *vtaA* negative isolates were PAGE type I, while all group 1 *vtaA* positive isolates were classified as PAGE type II. All serovars 2, 5, 7, 13 isolates were group 1 *vtaA* positive and all serovars 8, 9, 10 isolates were group 1 *vtaA* negative *(Table 2)*.

Table 2. *Characteristics of the H. parasuis isolates in this study*

Characteristics	Serovars											
	1	2	4	5	6	7	8	9	10	13	15	NT
PAGE type I strains or Group 1 *vtaA* negative strains	1	0	1	0	15	0	13	8	21	0	12	9
PAGE type II strains or Group 1 *vtaA* positive strains	8	1	9	11	1	7	0	2	0	2	5	7
Serotypeable strains	9	1	10	11	16	7	13	10	21	2	17	16
Serotypeable strains frequency (%)	6.8	0.8	7.5	8.3	12.0	5.3	9.8	7.5	15.8	1.5	12.8	12.0

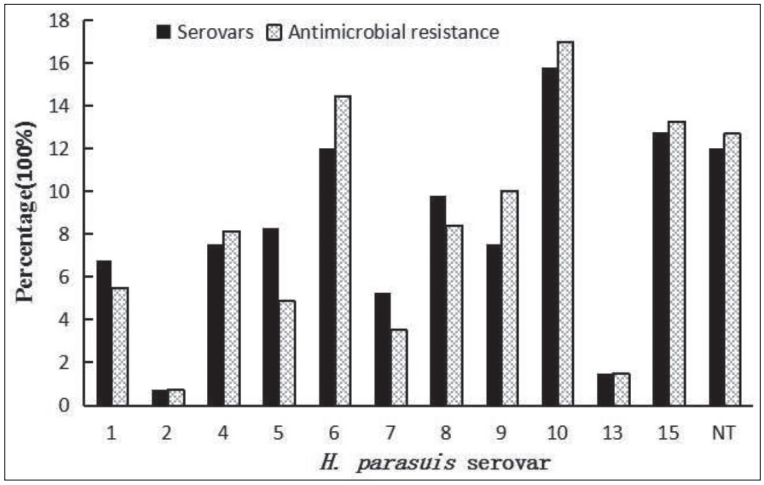
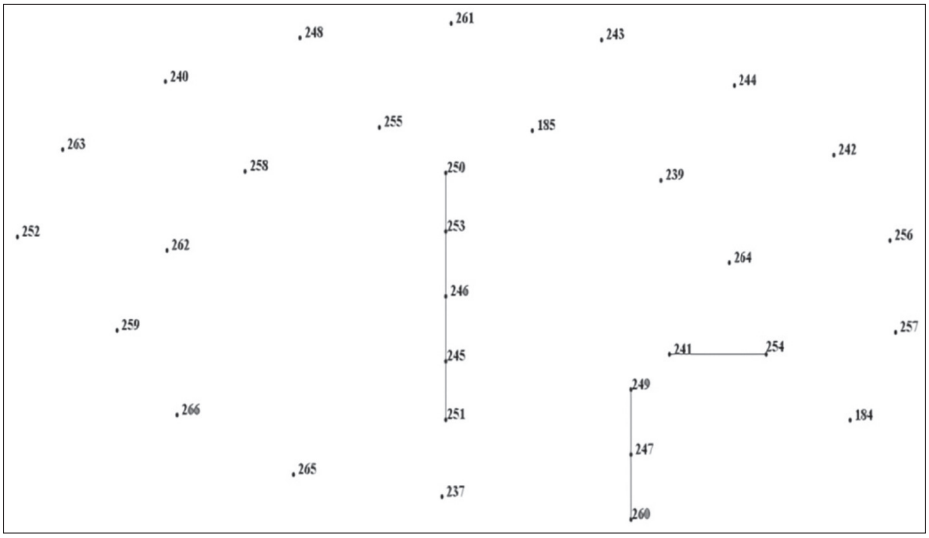


Fig 1. Distribution of *H. parasuis* serovars and antimicrobial resistance among each of these serovars.Serovars: frequency (%) = number of strains belonging to a certain serovar/total number of strains ×100%.Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain serovars/total number of resistances to all tested antimicrobial agents across all strains×100%

Fig 2. Analysis of 31 *H. parasuis* STs in the study using eBURST



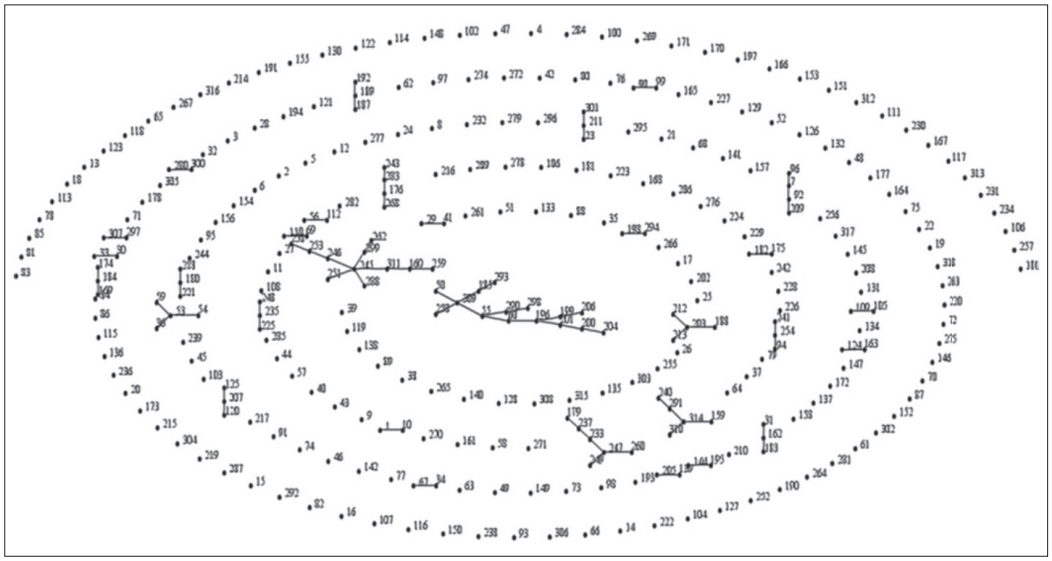


Fig 3. Analysis of all *H. parasuis* STs from the MLST database of *H. parasuis* using eBURST

In this study, we identified 13 new alleles across five of the seven housekeeping genes. Five new alleles were identified in 6 *pgd* (55-59), two in *atpD* (31-32), one in *frdB* (48), four in *infB* (51-54), and one in *mdh* (42). A total of 31 STs were found in this study, and 28 STs (ST239-ST266) were new MLST STs. clonal complex (CC)1 comprised ST241 and ST254; CC2 comprised ST245, ST246, ST250, ST251, and ST253; and CC3 comprised ST247, ST249

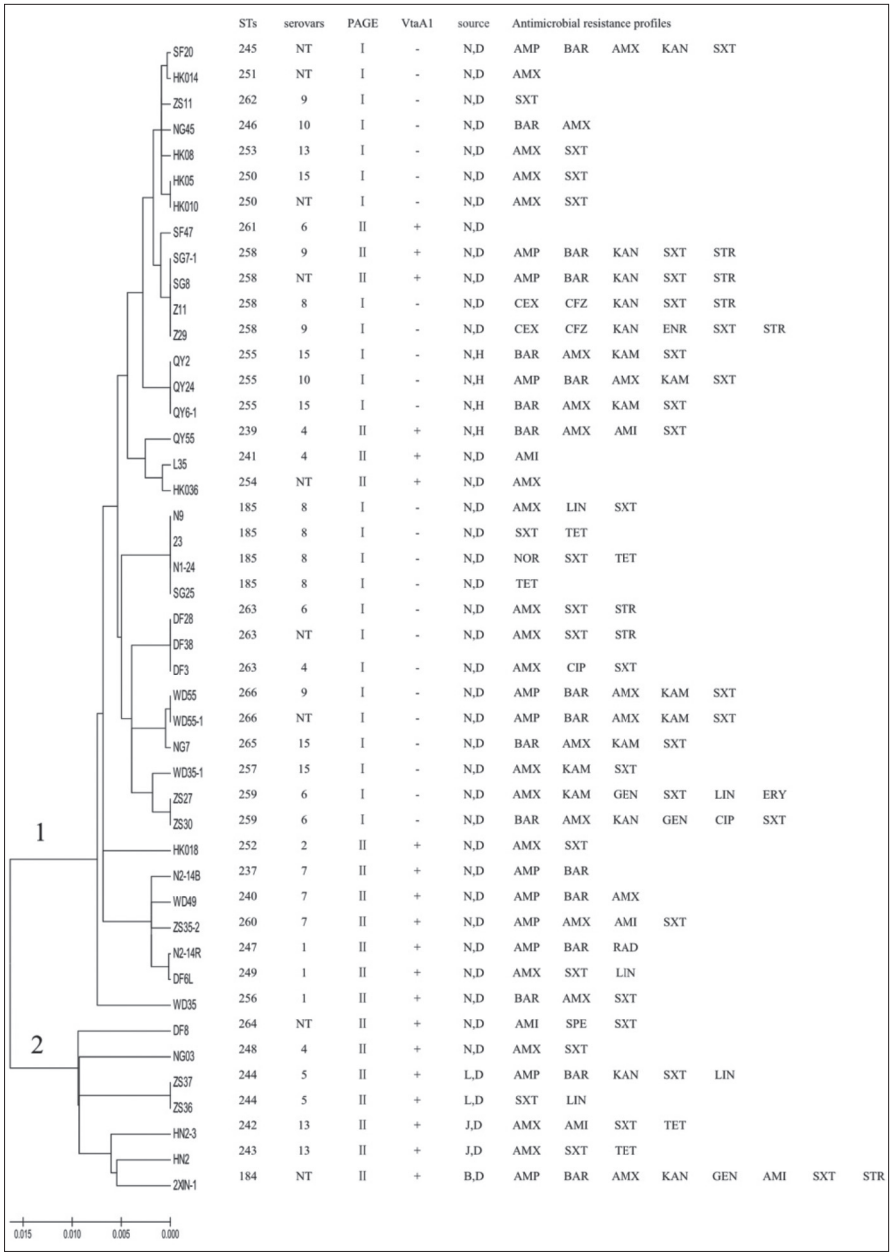


Fig 4. Neighbor-Joining tree derived from MLST target sequences of 45 *H. parasuis* strains. AMP: Ampicillin, BAR: Carbenicillin, AMX: Amoxicillin, CFZ: Cefazolin, CEX: Cefalexin, CXM: Cefuroxime, KAN: Kanamycin, GEN: Gentamicin, AMI: Amikacin, SPE: Spectinomycin, ENR: Enrofloxacin, NOR: Norfloxacin, CIP: Ciprofloxacin, SXT: Trimethoprim + Sulfamethoxazole, LIN: Lincomycin, ERY: Erythromycin, TET: Tetracycline, STR: Streptomycin; N: nasal, B: blood, J: joint, L: lung, H: healthy pig, D: diseased pig

and ST260 *(Fig. 2)*. ST245 was the predicted founder.

We also analyzed the population structure based on 318 STs from the MLST database of *H. parasuis*. The eBURST organized the 318 STs into 31CCs and 209 singletons. The predicted founders of CC1, CC2, CC4, CC6, CC9, CC13, CC14 and CC16 were ST309, ST245, ST314, ST203, ST211, ST180, ST184 and ST207, respectively. The predicted founders of CC3, CC5, CC7, CC10 and CC11 were multiple candidates. There were no predicted founders in other CCs *(Fig. 3)*.

The *sodA* gene of 45 *H. parasuis* isolates were sequenced. The results identified only 7 *H. parasuis* isolates (HN2-3, HN2, 2XIN-1, ZS36, ZS37, DF8 and NG03) with all four highly pathogenic sites of amino acids: aspartic acid at position 16 (Asp-16), leucine at position 49 (Leu-49), arginine at position 69 (Arg-69) and isoleucine at position 186 (Ile-186), other isolates with 1 to 2 changes at position 16, 49 and 69.

A neighbour-joining tree derived from the MLST target sequences of 45 *H. parasuis* isolates was constructed. Two major clades were obtained (clade 1and clade 2). Clade 1 includes the majority of isolates (84.4%) and STs (80.6%), including serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and some of the NT isolates. Clade 2 includes a minority of the isolates (15.6%) and STs (19.4%), including serovars 4, 5, 13 and some NT isolates *(Fig. 4)*. These isolates allocated to clade 2 all classified as PAGE type II (whole-cell protein profiling), contained all four highly pathogenic amino acids sites *(sodA* sequencing), and were positive for *vtaA* groups 1 *(vtaA* characterization). However, 65.8% of the 45 isolates allocated to clade 1 classified as PAGE type I and were negative for *vtaA* groups 1 *(Fig. 4)*.

DISCUSSION

This work was based on 133 *H. parasuis* isolates from southern China. These isolates belonged to eleven serovars (1, 2, 4-10, 13 and 15) with 13% of them being characterized as NT. Previous studies have reported that different *H. parasuis* isolates may belong to the same MLST ST, even if they consist of different serovars. Olvera et al.[21] and Olvera et al.[22] found that although *H. parasuis* isolates CD7-3 (serovar 14), CD9-1(serovar15), and CD10-4 (serovar 10) belonged to different serovars, they were all assigned to *H. parasuis* ST 46. Similarly, Wang et al.[11] found that although *H. parasuis* isolates H33 (NT), H35 (serovar 15), and H36 (serovar 14) belonged to different serovars, they were all assigned to *H. parasuis* ST 181. In this study, we found a similar result *(Fig. 4)*, which illustrates a lack of correlation between the *H. parasuis* STs and serovars.

In this study, 28 out of the 31 STs identified with the MLST method were novel. The eBURST organized the 31 STs into 3 CCs and 21 singletons, and organized all STs from the MLST database of *H. parasuis* into 31 CCs and 209 singletons, which illustrates the high heterogeneity of the population structure of *H. parasuis*.

In China, Zhou et al.[23] reported that 44.5% and 70.9% of 110 isolates were resistant to trimethoprim + sulfamethoxazole and enrofloxacin, respectively. Xu et al.[24] reported that most of the tested 112 isolates in theirs study were resistant to nalidixic acid (84.8%), TMP (67.9%), trimethoprim + sulfamethoxazole (58%), enrofloxacin (45.5%) and ciprofloxacin (41.1%). Zhao et al.[25] reported that 82.5% and 55.9% of 143 isolates were resistant to nalidixic acid and enrofloxacin. In our study, we observed a relatively high level of resistance to trimethoprim + sulfamethoxazole (89.9%), tetracycline (75.3%), amoxicillin (69.1%), streptomycin (63.6%), carbenicillin (60.2%), kanamycin (46.6%), and ampicillin (45.6%). This high frequency of resistance may be attributed to the intensive use of these antibiotics in the hog industry in China. We compared the distribution of serovars with the distribution of the incidence of antimicrobial resistance, and concluded that there was no statistically significant correlation between the antibiotic resistance and the serovars (x^2-test, $P>0.05$).

The association between drug resistance and the *H. parasuis* MLST STs was ambiguous. For example, although the isolates of N9, N1-24, 23 and SG25 were isolated from different farms and belonged to the same ST (ST185), they had different resistances *(Fig. 4)*. Similarly, the isolates ZS27 and ZS30, isolated from same farm and assigned to the same ST (ST259), also had different drug resistance patterns.

The isolates allocated to PAGE type II (whole-cell protein profiling), group 1 *vtaA* positive *(vtaA* characterization) and clade 2 (based on MLST target sequences) are potentially virulent strains of *H. parasuis*; whereas the isolates allocated to PAGE type I, group 1 *vtaA* negative and clade 1 are generally avirulent [7,9-11]. In the study, the majority of the isolates were classified as 'non-pathogenic, one explanation for this finding can be that the majority of the isolates (88%) were isolated from the nasal cavity. The isolates allocated to clade 2 all classified as PAGE type II and group 1 *vtaA* positive, these isolates showed the molecular characteristics of highly pathogenic strains in whole-cell protein profiling, *vtaA* groups 1 and MLST.

Previous research has suggested that the mutations of in *sodA* amino acid sites can influence the activity of *sodA* [26,27]. Le [19] further reported there are four changs in *sodA* amino acid sites between virulent and avirulent strains of *H. parasuis*. In the study, the isolates allocated to clade 2 (potentially virulent isolates) contained all four highly pathogenic amino acids sites in *sodA*, the isolates allocated to clade 1 (potentially avirulent isolates) were identified with 1 to 2 mutations in four highly pathogenic amino acids sites in *sodA,* which illustrates the *sodA* sequence may be uesd to predict the virulence of *H. parasuis* isolates, which requires further testing.

In summary, the current findings illustrates a high levels of drug resistance in *H. parasuis* isolates in Southern China, a high heterogeneity of the population structure of *H. parasuis*, a lack of correlation between the *H. parasuis* STs and serovars, there was no statistically significant correlations between antimicrobial resistance and the serovars and the *sodA* sequence may be uesd to predict the virulence of *H. parasuis* isolates, just as whole-cell protein profiling, *vtaA* groups 1 and MLST can be uesd to predict the virulence.

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