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Characterization of antimicrobial resistance genes in *Haemophilus parasuis* isolated from pigs in China

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Background: *Haemophilus parasuis* is a common porcine respiratory disease that causes high rates of morbidity and mortality in farmed swine. We performed a molecular characterization of antimicrobial resistance genes harbored by *H. parasuis* from pig farms in China.

Methods: We screened 143 *H. parasuis* isolates for the presence of 64 antimicrobial resistance genes by PCR amplification and DNA sequence analysis. We determined quinolone resistance determining region mutations of DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE). The genetic relatedness among the strains was analyzed by pulsed-field gel electrophoresis.

Results: We found 14 antimicrobial resistance genes were present in these isolates, including *TEM*-1, *ROB*-1.*ermB,ermA ,flor, catl,tetB,tetC, rmtB,* rmtD, aadA1, aac(3')-IIC, *sul1,* and *sul2* genes. Interestingly, one isolate carried 5 antibiotic resistance genes (*tetB, tetC, flor, rmtB, sul1*). The genes *tetB*, *rmt*B, and *flor* were the most prevalent resistance genes in *H. parasuis* in China. Alterations in the *gyrA* gene (S83F/Y, D87Y/N/H/G) were detected in 81% of the strains and *parC* mutations were often accompanied by a *gyrA* mutation. pulsed-field gel electrophoresis typing revealed 51 unique patterns in the isolates carrying antibiotic resistance genes indicating considerable genetic diversity and suggesting the genes were spread horizontally.

Discussion: The current study demonstrated that the high antibiotic resistance of *H. parasuis* in piglets is a combination of transferable antibiotic resistance genes and multiple target gene mutations. *Gyr*A gene mutation also was the most important role in quinolone resistance. These data provide novel insights for the better understanding of the prevalence and epidemiology of antimicrobial resistance in *H. parasuis*.

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Abstract

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Introduction

Haemophilus parasuis is the etiological agent of Glässer's disease that causes significant morbidity and mortality as well as economic losses in the global pig industry(Oliveira & Pijoan 2004). Antimicrobial therapy is used to prevent and control this infection even though antimicrobial agents are also used for growth promotion in pigs(Lancashire et al. 2005). However, extended agricultural use of antibiotics poses a risk for selecting antibiotic resistant pathogens and antibiotic resistance in *H. parasuis* is increasing(Aarestrup et al. 2004; de la Fuente et al. 2007; Markowska-Daniel et al. 2010; Walsh & Fanning 2008; Wissing et al. 2001; Xu et al. 2018). In China, the resistance rate of *H. parasuis* to antimicrobials is also increasing resulting in limited therapeutic choices(Zhou et al. 2010).

Increases in antibiotic resistance among bacteria is most often the result of antibiotic resistance gene (ARG) transfer mediated by mobile DNA elements such as plasmids, transposons and integrons in Gram-negative bacteria(Lancashire et al. 2005; San Millan et al. 2007). A long history of antibiotic use in the swine industry has generated a strong selective pressure for resistance transfer mediated by plasmids and transposons within and between bacterial species. Plasmids play a key role in this process by acting as vehicles for horizontal gene transfer(San Millan et al. 2016). The most prominent ARG types associated with resistance in *H. parasuis* include *ROB-1, tetB, tetL, qnrA1, qnrB6, aac(6')-Ib-cr, lnu(C)* and *flor(Dayao et al. 2016; Guo et al. 2011; Kehrenberg et al. 2005; Lancashire et al. 2005; Li et al. 2015; San*

Millan et al. 2007). In China, *ROB-1, qnrA1, qnrB6, aac(6')-Ib-cr, lnu(C)* and *flor* have been identified in *H. parasuis(Guo et al. 2012; Guo et al. 2011; Li et al. 2015)*. Horizontal gene transfer of ARG-carrying mobile elements and vertical gene transfer by the proliferation of ARG hosts facilitate resistance spread(Xu et al. 2018). Moreover, quinolone resistance determining region mutations(QRDR) of *gyr*A and *par*C were related to resistance. Therefore, studying ARG fates and their horizontal and vertical transfer-related elements and QRDRs can provide a comprehensive insight into resistance mechanisms.

H. parasuis is one of the most important respiratory pathogens in pigs(Guo et al. 2012; Zhang et al. 2014; Zhou et al. 2010), so more information is needed on the characterization of resistance genes associated with the increase in antibiotic resistance for this bacterium. In the present study, we examined resistance determinants, QRDRs and genetic relatedness in *H. parasuis* strains from pig farms in China.

Materials and Methods

Bacterial isolates

143 *H. parasuis* strains were isolated from diseased swine suffering polyserositis, pneumonia or meningitis between February 2014 and March 2017 from China, have been identified. The study was approved by the animal research committees of the South China Agriculture University(No.2014-025，see supplemental S1). All procedures were performed to minimize animal suffering as defined by the guidelines issued by this committee.

Fluoroquinolone antimicrobial susceptibility testing

Nalidixic acid, ciprofloxacin, levofloxacin, enrofloxacin, norfloxacin and lomefloxacin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Minimal inhibitory concentrations (MIC) were determined in fastidious medium consisting of tryptic soy broth (TSB) (OXOID, UK) with 5% bovine serum and 10 μg/mL nicotinamide adenine dinucleotide (NAD) (Sigma, Inc., USA) in 96-well microtiter plates. All plates were inoculated following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using Table 2E *Haemophilus influenzae* and *Haemophilus parainfluenzae* M02 and M07(CLSI 2015). The plates were incubated in an atmosphere containing 5% CO_2 at 37°C for 24 h. The MIC value was defined as the lowest concentration resulting in no visible bacterial growth. The reference strains *Haemophilus influenzae* ATCC 49247 and *Escherichia coli* ATCC 25922 served as quality controls for MIC determinations.

ARGs and integrons detection

DNA was extracted from whole organisms using the quick boiling method(Sambrook & Russell 2001). PCR assays were used to screen for the presence of 64 ARG types including resistance to quinolones, β-lactams, macrolides, tetracycline, aminoglycosides, chloramphenicol, sulfonamides as well as for the integrase gene (Table 1). Purified PCR products were directly sequenced from both ends or cloned into plasmid vector pMD18-T, and then sequenced. DNA sequence similarity searches were performed against the GenBank database using BLAST software to confirm gene identity.

Detection of mutations in QRDRs of *gyrA, gyrB, parC, and parE*

Mutations in the quinolone resistance determining regions (QRDR) mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes were identified after DNA sequencing of PCR products generated with the primers listed in Table 2.

Pulsed-field gel electrophoresis

Genetic relatedness of *H. parasuis* strains carrying ARGs was determined by pulsed field electrophoresis (PFGE) of *Cpo*I- (TaKaRa, China) digested genomic DNA samples(Zhang et al. 2011). PFGE typing used a CHEF Mapper electrophoresis system (Bio-Rad, USA) with 2.16–63.8s for 21 h. *Salmonella enterica* serovar Braenderup H9812 DNA digested with *CpoI* was used for a size standard. Interpretation of the PFGE patterns was accomplished using BioNumerics 6.6 software (Applied Maths, USA)(Tenover et al. 1995).

Results

The prevalence of resistance genes

In the current study, we examined 143 *H. parasuis* strains and 16 (11.2%) carried β-lactamases including *TEM*-1 and *ROB*-1. Tetracycline resistant strains carried *tet*B and *tet*C. There were two isolates (1.40%) also yielded the erythromycin resistance genes: 1 for *erm*A, and 1 for *erm*B. A higher proportion (16.1%) carried chloramphenicol resistance genes including 10 c*atl* and 13 *flor.* Aminoglycoside resistance was also high (11.9%) and included the genes *rmtB*,

rmtD, *aadA1* and *aac*(3')-IIC. The sulfonamide resistance genes were represented by *sul*1 and *sul*2 and were found in 9 (6.3%) and 2 (1.4%) of the isolates, respectively (Table 3).

The resistance gene patterns were diverse and 39 isolates carried one gene, 24 carried two and 9 isolates carried three genes. Interestingly, strain HP142 carried five genes *tetB, tetC, flor, rmtB* and *sul1*. Overall, *tet*B, *rmt*B and *flor* were the most prevalent resistance genes in these *H. parasuis* isolates from Chinese pig farms (Table 4). Other genes were not detected in this study.

QRDRS

We also identified several QRDR mutations among the resistant *H. parasuis* strains. Mutations in *gyrA* (S83F/Y, D87Y/N/H/G) were detected in 116 (81%) of the strains. In addition, 79 strains had *parC* mutations (L379I/ Y557C/ V648I/E678D) and most of these were accompanied by *gyrA* mutations. Only 9 strains had single *parC* mutations that were either L379I, Y557C, E678D, L379I or Y557C. The strains with *gyrA* mutations at either codon 83 or 87 showed higher MIC values compared with the 18 strains lacking mutations. The MIC values of the strains with single *parC* mutations were not significantly different from controls. No mutations were found in *gyrB* and *parE* (Table 5).

PFGE

The 73 *H. parasuis* strains carrying resistance determinants were typed by PFGE and were genomically heterogenic. We identified 51 unique *CpoI* patterns but no evidence of clonality (Figure 1).

Discussion

There have been few complete and systematic molecular studies of antimicrobial resistance in *H. parasuis*. The genes *ROB-1, tetB, tetL, qnrA1, qnrB6, aac(6')-Ib-cr, lnu(C)* and *flor* were the only that were previously identified and that correlated with resistance(Dayao et al. 2016; Guo et al. 2011; Kehrenberg et al. 2005; Lancashire et al. 2005; Li et al. 2015; San Millan et al. 2007). Cephalosporinases, which are naturally present in some enterobacterial species, can be mobilized by transposons and migrate *via* plasmids into other species. Moreover, the abuse of antimicrobial agents increases the number of carbapenem-resistant strains generating a public health concern(Yang et al. 2017). In the Enterobacteriaceae, theTEM-1 β-lactamase is the predominant genotype(Yang et al. 2017). In our study, we identified both *TEM-1* and *ROB-1* β-lactamase genes which are widespread among *H. parasuis* and *Pasteurella spp(Guo et al. 2012; San Millan et al. 2007)*. *TEM-1* and ROB-1 are usually present in *H. influenzae* and have particularly geographic distributions in different countries(Farrell et al. 2005). These geographic differences may also be present in *H. parasuis*. The first reports of *TEM-1* and *ROB-1* were in China and Spain, respectively(Guo et al. 2012; San Millan et al. 2007). *ROB-1* was located on plasmid pB1000 and recently a novel 2,661bp plasmid (pJMA-1) bearing *ROB-1* has been identified.

158 This plasmid possessed a backbone found in small Pasteurellaceae plasmids and was 100%
159 stable with a lower biological cost than pB1000(Moleres et al. 2015).
160
161 We also identified genes encoding tetracycline efflux pumps (*tetB* and *tetD*) in this study. The
162 first tetracycline resistant gene identified in *H. parasuis* was *tetB* and this gene is the most
163 common tetracycline resistance gene in *Actinobacillus pleuropneumoniae* and *Pasturella*
164 *multocida(Dayao et al. 2016; Matter et al. 2007)*. The genes *tetH* and *tetM* are present in other
165 members of the Pasteurellaceae(Roberts 2012). Furthermore, the *tetB*-carrying plasmid pHS-Tet
166 in *H. parasuis* was similar to a *tetL*-carrying plasmid in *Pasteurella* isolates(Kehrenberg et al.
167 2005; Lancashire et al. 2005). This is the first report of the *tetD* gene in *H. parasuis* isolates from
168 China and needs further study. Tetracycline resistance genes are often associated with
169 conjugative and mobile genetic elements enabling horizontal transfer(Dayao et al. 2016; Roberts
170 2012). The presence of *tetD* suggests that tetracycline resistance in *H. parasuis* relies on efflux
171 pumps.
172
173 In bacteria with animal origins, five florfenicol resistance genes (*floR, fexA, fexB, cfr* and
174 *optrA*) have been reported(Schwarz et al. 2004; Wang et al. 2015). In Gram-negative bacteria,
175 *floR* makes the greatest contribution to florfenicol resistance and this has been described for a
176 number of bacterial species(He et al. 2015; Meunier et al. 2010; Schwarz et al. 2004; Wang et al.
177 2015). The emergence of florfenicol resistance in *H. parasuis* isolates was attributable to a novel

178 small plasmid pHPSF1 bearing *floR*. This novel plasmid was similar to other Pasteurellaceae
179 plasmids suggesting these species prefer to exchange genetic elements with each other.
180
181 High-level aminoglycoside resistance mediated by the production of the 16S rRNA
182 methylases *armA, rmtA* to *H* and *npmA,* and resistance is increasing among Gram-negative
183 pathogens(Du et al. 2009). However, until now, few studies have described the presence of the
184 *armA* and *rmtB* genes in food animals. The strains in our study also carried *rmtB*, *rmtD*, *aad*A1
185 and *aac* (3') IIc and these warrants further investigation.
186
187 The macrolide-resistance genes *erm*A and *erm*B showed a low frequency in our *H. parasuis*
188 isolates. These genes are responsible for ribosomal binding site modifications that are the most
189 important macrolide resistance mechanisms(Takaya et al. 2010).
190
191 The *sul*1, *sul*2 and *sul*3 genes are dihydropteroate synthases involved in sulfonamide
192 resistance of Gram-negative bacteria and are usually associated with an integron system and a
193 conjugative plasmid(Vo et al. 2006). In the current study, we identified both *sul*1 and *sul*2, and
194 these genes most likely accounted for the observed resistance to trimethoprim-sulfamethoxazole.
195 These results are similar to others in Gram-negative bacteria(Koljalg et al. 2009; Matter et al.
196 2007).
197

198 The *qnr* genes that enable quinolone resistance have been identified in many Gram-negative
199 bacteria worldwide(Cao et al. 2017)]. Most of these genes are located on a transposon-like
200 sequence or an integron on a conjugative plasmid that facilitates rapid spread of fluoroquinolone
201 resistance(Cao et al. 2017). Integrons are a novel antibiotic resistance mobile element and
202 function as a primary source of antibiotic genes in the form of gene cassettes(Chirila et al. 2017).
203 However, no *qnr* genes or integrons were detected in our study. Their absence suggested that
204 quinolone resistance was conferred by other genes or through another resistance mechanism.
205
206 This is the first report describing the presence of the *tetC, sul1*, *sul2*, *ermA*, *ermB*, *catl*, *rmtB,*
207 *rmtD*, *aad*A1 and *aac* (3')-IIC genes in *H. parasuis,* to the best of our knowledge. Nevertheless,
208 we did find several isolates with reduced antibiotic susceptibility that did not harbor any of the
209 tested resistance genes. This suggests that *H. parasuis* possesses other resistance mechanisms
210 such as mutations, decreases in permeability and increases in efflux pump activity or yet
211 unknown antibiotic resistance mechanisms. In addition, the widespread dissemination of
212 resistance genes and integrons could potentially fuel the rapid development of antimicrobial
213 resistance due to their high transfer capabilities(Hussein et al. 2009). Therefore, more study is
214 needed on this subject.
215
216 There have been numerous studies demonstrating *gyrA* and *parC* mutations engendering
217 fluoroquinolone resistance in Gram-negative bacteria such as *Salmonella spp.* and *E. coli(Cao et*
218 *al. 2017)*. In *H. parasuis*, the *gyrA* mutations S83Y, S83F, D87Y, D87N and D87G are

219 correlated with fluoroquinolone resistance. In addition, the *parC* mutations Y577C, V648I,
220 E678D, S669F, A464V and A466S and *parE* mutations S283G, A227T and G241S were also
221 found in these strains(Guo et al. 2011). In another study, mutations of *gyrA* D87N, *parC* S73R
222 and *parE* T551A were involved in fluoroquinolone resistance, but other mutations such as in
223 *gyrA* (452D^V/G, 627G^E), *gyrB* (211V^I, 254D^G), *parC* (73S^R/I, 227Q^H, 379L^I,
224 578C^Y) and *parE* (551T^A) occurred less frequently(Zhang et al. 2013). However, the *parE*
225 mutation in *A. pleuropneumoniae* is possibly not involved in enrofloxacin resistance(Wang et al.
226 2010). In our study, most strains possessed *gyrA* mutations, and six strains possessed a *gyrA*
227 mutation (D87H) not been previously reported. However, we do not know whether this mutation
228 is directly related to fluoroquinolone resistance. We also identified four *parC* mutations. Unlike
229 other studies, we found the *parC* 578 mutation in both resistant and sensitive strains suggesting
230 this mutation is not involved in resistance(Zhang et al. 2013). Overall, the QRDR analysis in our
231 study suggested that the mutations at codon 83 or 87 of *gyrA* were responsible for
232 fluoroquinolone resistance and that *gyrB* and *parE* were not.
233
234 Interestingly, our PFGE results indicated that almost 70% of our *H. parasuis* were genetically
235 diverse, similar to a recent report(Guo et al. 2012). These results are in contrast to a previous
236 study presenting evidence for the clonal spread of β-lactam resistance(San Millan et al. 2007).
237 Our data suggests that resistance genes are spread *via* transferable elements such as plasmids and
238 transposons in addition to clonal spread. Therefore, research on mechanisms for the spread of
239 antimicrobial resistance in *H. parasuis* needs further investigation.

240

241 **Conclusions**

242 In this study, we comprehensively and systematically investigated for the first time the
243 distribution of the most common resistance genes in *H. parasuis* in China. These genes included
244 *tetB*, *tetC, sul1*, *sul2*, *ermA*, *ermB*, *TEM-1*, *ROB-1*, catl, flor, rmtB, rmtD, aadA1 and aac (3')-
245 IIC. The *gyrA* mutations S83F/Y and D87Y/N/H/G correlated with fluoroquinolone resistance in
246 *H. parasuis*. These strains were also genetically diverse as judged by PFGE. These data suggest
247 that antimicrobial resistance in *H. parasuis* is primarily the result of transferable determinants
248 and multiple target gene mutations. The exact roles for these detected resistance determinants in
249 *H. parasuis* await further study.

250

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252 We thank members of our laboratories for fruitful discussions.

253

254 **Supplemental information**

255 Ethics statement see supplemental S1.

256 Information of separation site, separation time and resistance gene for H. parasuis see
257 supplemental S2.

258

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413

Table 1(on next page)

Antibiotic resistance gene testing of the *H. parasuis* isolates in this study

Antibiotic	Resistance genes	Primers
quinolones	qepA, qnrA, qnrB, qnrC, qnrD, qnrS, oqxAB, *aac(6')-Ib-cr*	(Cavaco et al. 2009; Yang et al. 2017; Zhao et al. 2010)
β-lactams	TEM, rob-1, SHV, CTX-M-1G, CTX-M-9G, CTX-M-2G, CTX-M-64, CTX-M-25 DHA, *VIM-1, VIM-2, SPM-1, CMY-2, npmA, OXA, NDM, KPC, IMP, SPM, FOX*	(Grobner et al. 2009; Liu et al. 2007; San Millan et al. 2007; Weill et al. 2004)
tetracyclines	*tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetL-1, tetL-2*	(De Gheldre et al. 2003; Matter et al. 2007; Miranda et al. 2009)
aminoglycosides	*rmtB, rmtC, armA, rmtA, rmtD, aadB[ant(2'')-la], aacC2 [aac(3)-Iic], aacC4 [aac(3)-Iva], aadA1,aac(6)-31*	(Doi & Arakawa 2007; Matter et al. 2007)
macrolides	*ermA, ermB, ermC, mefA/E, fosA3*	(Hou et al. 2013; Matter et al. 2007; Sutcliffe et al. 1996)
chloramphenicol	*catl, cmlA, flor, cfr*	(Maka & Popowska 2016; Wang et al. 2015)
sulfonamides	*sul1, sul2, sul3, dfrA1， dfrB*	(Matter et al. 2007)
integrase gene	*intl1， intl2， intl3*	(Shibata et al. 2003)

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Table 2(on next page)

PCR primer sequences used to amplify QRDR genes

Gene	Primers	Sequence (5'-3')	Size (bp)	Reference
gyrA	GyrA-F	AGCGTTACCAGATGTGCGAGATG	620	This study
	GyrA-R	TTGCCACGACCTGTACGATAAGC		
gyrB	GyrB-F	TACATACGCTGTAGGTTCAAGGA	500	This study
	GyrB-R	CAAGATAATACGGAAATGGAGC		
parC	ParC-F	AACTTCAACATTACCACTTAGCCCTC	1445	This study
	ParC-R	G TACCTCACCAAGCCTCGCCATCT		
parE	ParE-F	CGATAATTCCCTTGAAGTCGTTG	609	This study
	ParE-R	ATTGATCTGCTCGCCACCCTCTG		

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Table 3(on next page)

Prevalence of ARG types isolated from *H. parasuis*

Gene	Number identified	Prevalence (%)
ermA	1	0.70
ermB	1	0.70
catl	10	6.99
flor	13	9.09
tetB	34	23.78
tetC	5	3.50
rmtD	1	0.70
rmtB	17	11.89
aadA1	4	2.80
aac(3')- IIC	6	4.20
sul1	9	6.29
sul2	2	1.40
TEM-1	9	6.29
rob-1	7	4.90

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Table 4(on next page)

Resistance gene patterns and the number of resistant strains

Pattern	No. of isolates	Isolate
ermA	1	HP016
ermB	1	HP018
tetB	16	HP008, HP020, HP044, HP050, HP054, HP068, HP071, HP072, HP076, HP082, HP085, HP097, HP098, HP109, HP120, HP135
catl1	1	HP035
tetC	1	HP051
rmtB	6	HP001, HP037, HP061, HP079, HP096, HP111
flor	4	HP069, HP121, HP123, HP127
sul1	2	HP075, HP131
aadA1	1	HP032
aac(3')-IIC	1	HP039
TEM-1	4	HP012, HP022, HP063, HP116
rob-1	1	HP073
catl1+rob-1	1	HP133
tetB+flor	2	HP026, HP053
tetB+aadA1	1	HP067
tetB+rob-1	1	HP112
tetB+tetC	1	HP017
tetC+flor	1	HP060
flor+aadA1	1	HP104
catl1+tetB	3	HP059, HP091, HP095
rmtB+ aac(3')-IIC	1	HP118
rmtB+TEM-1	3	HP065, HP078, HP140
rmtB+sul1	4	HP056, HP080, HP134, HP141
sul1+ aac(3')-IIC	2	HP040, HP066
sul2+rob-1	1	HP011
sul2+tetB	1	HP019
rob-1+aadA1	1	HP102
catl1+tetB+TEM-1	1	HP137
catl1+tetB+rob-1	2	HP013, HP094
catl1+tetB+flor	1	HP103
catl1+tetB+aac(3')-IIC	1	HP025
tetB+flor+rmtB	1	HP108
tetB+flor+ aac(3')-IIC	1	HP029

tetB+tetC+flor	1	HP113
rmtD+rmtB+TEM-1	1	HP117
tetB+tetC+flor+rmtB+sul1	1	HP142

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Table 5(on next page)

QRDR mutations and antibiotic MIC values for 143 *H. parasuis* isolates

QRDR mutation		Number of strains	MICs (mg/L)					
gyrA	*parC*		Nalidixic acid	Levofloxacin	Ciprofloxacin	Enrofloxacin	Norfloxacin	Lomefloxacin
—	—	18	0.25-128	<0.25–2	<0.25–4	<0.25–2	<0.25–4	<0.25–1
S83F/Y	—	8	1–>512	0.25–16	0.25–16	<0.25–8	0.25–256	<0.25–4
S83F/Y, D87Y/N/H/G	—	38	4–>512	0.25–32	1–>512	0.25–32	0.25–>512	0.25–64
S83F/Y	[a]L379I/Y557C/V648I	20	32–>512	0.25–64	0.25—32	<0.25–32	0.25—16	<0.25–128
D87Y/H	[b]L379I/Y557C	2	4, 16	0.25, 0.5	0.25, 0.5	2	1, 4	0.25, 0.5
S83F/Y, D87Y/N/Y/G/H	[c]L379I/Y557C/V648I/E678D	48	1–>512	2–128	2-64	0.25–32	0.25–>512	<0.25–64
—	[d] L379I/ Y557C/ L379I, Y557C, E678D/ L379I, Y557C	9	0.5–>512	0.25–8	0.25–16	0.5–16	0.25–>512	0.5–64

Mutation mode

[a] L379I; L379I+ Y557C+V648I; Y557C+ V648I; L379I+ Y557C; L379I+V648I

[b] L379I +Y557C; L379I

[c] L379I; Y557C; L379I+Y557C+V648I; L379I+Y557C+E678D; L379I+Y557C; Y557C+V648I

[d] L379I; Y557C; L379I+ Y557C+E678D; L379I+Y557C.

Figure 1(on next page)

Dendrogram of patterns generated by PFGE of 73 ARG-containing *H. parasuis* isolates