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

Yongda Zhao[1], Lili Guo[2], Jie Li[1], Xianhui Huang[1] and Binghu Fang[1]

[1] College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong, China
[2] Qingdao Yebio Biological Engineering Co., Ltd, Qingdao, Shandong, China

ABSTRACT

Background. *Haemophilus parasuis* is a common porcine respiratory pathogen 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 antimicrobial susceptibility against six fluoroquinolone antibiotics testing by the broth microdilution method, and 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. Susceptibility test showed that all isolates were low resistance to lomefloxacin (28.67%), levofloxacin (20.28%), norfloxacin (22.38%), ciprofloxacin (23.78%), however, high resistance levels were found to nalidixic acid (82.52%) and enrofloxacin (55.94%). In addition, we found 14 antimicrobial resistance genes were present in these isolates, including $bla_{\text{TEM-1}}$, $bla_{\text{ROB-1}}$, *ermB, ermA, flor, catl, tetB, tetC, rmtB, rmtD, aadA1, aac(3′)-llc, sul1, and sul2* genes. Interestingly, one isolate carried five antibiotic resistance genes (*tetB, tetC, flor, rmtB, sul1*). The genes *tetB*, *rmtB,* 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 high-level antibiotic resistance genes, indicating considerable genetic diversity and suggesting that 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. These data provide novel insights for the better understanding of the prevalence and epidemiology of antimicrobial resistance in *H. parasuis*.

Subjects Microbiology, Molecular Biology, Epidemiology, Statistics
Keywords Antimicrobial resistance genes, QRDR, *Haemophilus parasuis*, PRGE

Submitted 1 November 2017
Accepted 23 March 2018
Published 9 April 2018

Corresponding author
Binghu Fang, fangbh@scau.edu.cn

Academic editor
Sheng Chen

Additional Information and Declarations can be found on page 12

DOI **10.7717/peerj.4613**

How to cite this article Zhao et al. (2018), Characterization of antimicrobial resistance genes in *Haemophilus parasuis* isolated from pigs in China. **PeerJ 6:e4613; DOI 10.7717/peerj.4613**

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, Seyfarth & Angen, 2004*; *De la Fuente et al., 2007*; *Markowska-Daniel et al., 2010*; *Walsh & Fanning, 2008*; *Wissing, Nicolet & Boerlin, 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 $bla_{\mathrm{ROB-1}}$, *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, $bla_{\mathrm{ROB-1}}$, *qnr*A1, *qnr*B6, *aac* (6′)-*Ib-cr, lnu*(C) and *flor* have been identified in *H. parasui* s (*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 *gyrA* and *parC* 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 strains

We isolated 143 *H. parasuis* strains from different diseased swine suffering polyserositis, pneumonia or meningitis between February 2014 and March 2017 in China. All strains were isolated from lung, brain, heart blood, pericardial effusion, pleural effusion, peritoneal effusion and joint fluid by aseptic inoculation ring, and cultured on tryptic soy agar (TSA) or tryptic soy broth (TSB) (Becton Dickinson, Owings Mills, MD, USA) containing 10 μg/ml nicotinamide adenine dinucleotide (NAD; Sigma, St. Louis, MO, USA) and

Table 1 **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	*bla*$_{TEM}$ − 1, *bla*$_{ROB-1}$, *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, Rodriguez & Galan-Vidal (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*	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)

5% bovine serum (Gibco, Auckland, New Zealand). Plates were incubated at 37 °C for 24–48 h. All isolates were identified by PCR (Angen et al., 2007). The study was approved (No.2014-025).

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 TSB with 5% bovine serum and 10 μg/mL NAD in 96-well microtiter plates. All plates were inoculated following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using *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 *H. 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 *gyr*A, *gyr*B, *par*C, and *par*E

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.

Table 2 **PCR primer sequences used to amplify QRDR genes.**

Gene	Primers	Sequence (5′-3′)	Size (bp)	Reference
gyrA	GyrA-F GyrA-R	AGCGTTACCAGATGTGCGAGATG TTGCCACGACCTGTACGATAAGC	620	This study
gyrB	GyrB-F GyrB-R	TACATACGCTGTAGGTTCAAGGA CAAGATAATACGGAAATGGAGC	500	This study
parC	ParC-F ParC-R	AACTTCAACATTACCACTTAGCCCTCG TACCTCACCAAGCCTCGCCATCT	1,445	This study
parE	ParE-F ParE-R	CGATAATTCCCTTGAAGTCGTTG ATTGATCTGCTCGCCACCCTCTG	609	This study

Pulsed-field gel electrophoresis

Genetic relatedness of *H. parasuis* strains carrying ARGs was determined by pulsed field electrophoresis (PFGE) of *CpoI* - (TaKaRa, Beijing, China) digested genomic DNA samples (*Zhang et al., 2011*). PFGE typing used a CHEF Mapper electrophoresis system (BioRad, Hercules, CA, USA) with 2.16–63.8 sfor 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, Sint-Martens-Latem, Belgium) (*Tenover et al., 1995*).

RESULTS

Bacterial strains analysis

In the current study, 143 *H. parasuis strains* were isolated and 73 carried antibiotic resistance genes. Information on isolation site, isolation time and resistance gene content are listed in Table 3.

Fluoroquinolone antimicrobial susceptibility testing

The results of the fluoroquinolone antimicrobial susceptibility of 143 *H. parasuis* isolates are listed in Supplemental Information 1. It showed that 82.52% and 55.94% of all isolates were resistant to nalidixic acid and enrofloxacin, respectively. Resistance of lomefloxacin, levofloxacin, norfloxacin, ciprofloxacin were 28.67%, 20.28%, 22.38%, 23.78%, respectively.

ARG and integron prevalence and detection

We examined 143 *H. parasuis* strains and 16 (11.2%) carried β-lactamases including $bla_{\mathrm{TEM-1}}$ and $bla_{\mathrm{ROB-1}}$. Tetracycline resistant strains carried *tetB* and *tetC*. There were two isolates (1.40%) also yielded the erythromycin resistance genes: 1 for *ermA*, and 1 for *ermB*. A higher proportion (16.1%) carried chloramphenicol resistance genes including 10 *catl* and 13 *flor*. Aminoglycoside resistance was also high (11.9%) and included the genes *rmtB*, *rmtD*, *aadA1* and *aac(3′)*- llc. The sulfonamide resistance genes were represented by *sul1* and *sul2* and were found in 9 (6.3%) and 2 (1.4%) of the isolates, respectively (Table 4).

The resistance gene patterns were diverse and 39 isolates carried one gene, 24 carried two and nine isolates carried three genes. Interestingly, strain HP142 carried five genes

Table 3 List *H. parasuis* strains with their separation site, date, organ and resistance gene.

Isolates	Separation site	Date	Organ	Resistance gene
HP001	Fujian	2016	lung	*rmtB*
HP008	Fujian	2015	nasal cavity	*tetB*
HP011	Meizhou	2014	pericardial effusion	*sul2*+ $bla_{\text{ROB}-1}$
HP012	Jinan	2017	lung	$bla_{\text{TEM}-1}$
HP013	Zengcheng	2016	nasal cavity	*catl1*+*tetB*+ $bla_{\text{ROB}-1}$
HP016	Laiyang	2015	brain	*ermA*
HP017	Dongguan	2015	joint fluid	*tetB*+*tetC*
HP018	Qingdao	2016	lung	*ermB*
HP019	Hebei	2017	lung	*sul2*+*tetB*
HP020	Jilin	2016	lung	*tetB*
HP022	Huadou	2015	lung	$bla_{\text{TEM}-1}$
HP025	Zengcheng	2016	joint fluid	*catl1*+*tetB*+*aac(3′)-IIc*
HP026	Guangxi	2014	lung	*tetB*+*flor*
HP029	Guangxi	2015	heart blood	*tetB*+*flor*+ *aac(3′)-IIc*
HP032	Chengde	2014	joint fluid	*aadA1*
HP035	Guangxi	2017	heart blood	*catl1*
HP037	Fujian	2015	nasal cavity	*rmtB*
HP039	Hebei	2015	pericardial effusion	*aac(3′)-IIc*
HP040	Jiangmen	2016	lung	*sul1*+ *aac(3′)-IIc*
HP044	Jiangsu	2014	lung	*tetB*
HP050	Jiangsu	2016	pleural effusion	*tetB*
HP051	Jiangsu	2014	heart blood	*tetC*
HP053	Yunnan	2016	lung	*tetB*+*flor*
HP054	Guangzhou	2016	lung	*tetB*
HP056	Zhucheng	2017	lung	*rmtB*+*sul1*
HP059	Guangxi	2016	lung	*catl1* +*tetB*
HP060	Guangxi	2015	heart blood	*tetC*+*flor*
HP061	Qingdao	2016	lung	*rmtB*
HP063	Hebei	2015	lung	$bla_{\text{TEM}-1}$
HP065	Qingyuan	2015	lung	*rmtB*+ $bla_{\text{TEM}-1}$
HP066	Guangxi	2016	lung	*sul1*+ *aac(3′)-IIc*
HP067	Qingdao	2015	lung	*tetB*+*aadA1*
HP068	Qingyuan	2015	heart blood	*tetB*
HP069	Hunan	2017	heart blood	*flor*
HP071	Hebei	2015	lung	*tetB*
HP072	Zhucheng	2017	nasal cavity	*tetB*
HP073	Zhuhai	2016	joint fluid	$bla_{\text{ROB}-1}$
HP075	Fujian	2014	pericardial effusion	*sul1*
HP076	Henan	2017	heart blood	*tetB*
HP078	Henan	2015	lung	*rmtB*+ $bla_{\text{TEM}-1}$
HP079	Jining	2016	lung	*rmtB*

(*continued on next page*)

Table 3 (*continued*)

Isolates	Separation site	Date	Organ	Resistance gene
HP080	Jinan	2014	joint fluid	*rmtB+sul1*
HP082	Qingdao	2016	lung	*tetB*
HP085	Liaoning	2016	lung	*tetB*
HP091	Shaoguan	2017	pericardial effusion	*catl1+tetB*
HP094	Hebei	2015	lung	*catl1+tetB+* bla_{ROB-1}
HP095	Huadou	2017	lung	*catl1+tetB*
HP096	Zhengzhou	2014	heart blood	*rmtB*
HP097	Hunan	2016	pericardial effusion	*tetB*
HP098	Hebei	2014	lung	*tetB*
HP102	Anyang	2015	lung	bla_{ROB-1}*+aadA1*
HP103	Hunan	2017	lung	*catl1+tetB+flor*
HP104	Jiangsu	2016	joint fluid	*flor+aadA1*
HP108	Guangxi	2016	lung	*tetB+flor+rmtB*
HP109	Zhaoqing	2017	heart blood	*tetB*
HP111	Jiangxi	2015	lung	*rmtB*
HP112	Sihui	2016	heart blood	*tetB+*bla_{ROB-1}
HP113	Henan	2015	lung	*tetB+tetC+flor*
HP116	Boluo	2014	lung	bla_{TEM-1}
HP117	Hebei	2017	heart blood	*rmtD+rmtB+*bla_{TEM-1}
HP118	Huizhou	2016	lung	*rmtB+aac(3′)-IIC*
HP120	Hebei	2016	lung	*tetB*
HP121	Hebei	2016	heart blood	*flor*
HP123	Anhui	2015	lung	*flor*
HP127	Jilin	2015	joint fluid	*flor*
HP131	Yunnan	2014	heart blood	*sul1*
HP133	Huizhou	2016	lung	*catl1+rob-1*
HP134	Zhucheng	2014	lung	*rmtB+sul1*
HP135	Shaoguan	2016	pleural effusion	*tetB*
HP137	Yangzhou	2015	lung	*catl1+tetB+* bla_{TEM-1}
HP140	Conghua	2014	heart blood	*rmtB+* bla_{TEM-1}
HP141	Yangzhou	2017	lung	*rmtB+sul1*
HP142	Henan	2016	lung	*tetB+tetC+flor+rmtB+sul1*

tetB, tetC, flor, rmtB and *sul1*. Overall, *tetB*, *rmtB* and *flor* were the most prevalent resistance genes in these *H. parasuis* isolates from Chinese pig farms (Table 5). Other genes were not detected in this study.

Mutations in QRDRs of *gyr*A, *gyr*B, *par*C, and *par*E

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 nine 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

Table 4 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
bla_{TEM-1}	9	6.29
bla_{ROB-1}	7	4.90

Table 5 Resistance gene patterns and the number of resistant strains.

Pattern	No. of isolates	Pattern	No. of isolates
ermA	1	*flor+aadA1*	1
ermB	1	*catl1+tetB*	3
tetB	16	*rmtB+ aac(3′)-IIc*	1
catl1	1	*rmtB+* bla_{TEM-1}	3
tetC	1	*rmtB+sul1*	4
rmtB	6	*sul1+ aac(3′)-IIc*	2
flor	4	*sul2+* bla_{ROB-1}	1
sul1	2	*sul2+tetB*	1
aadA1	1	bla_{ROB-1}*+aadA1*	1
aac(3′)-IIc	1	*catl1+tetB+* bla_{TEM-1}	1
bla_{TEM-1}	4	*catl1+tetB+* bla_{ROB-1}	2
bla_{ROB-1}	1	*catl1+tetB+flor*	1
catl1+bla_{ROB-1}	1	*catl1+tetB+aac(3′)-IIc*	1
tetB+flor	2	*tetB+flor+rmtB*	1
tetB+aadA1	1	*tetB+flor+ aac(3′)-IIc*	1
tetB+ bla_{ROB-1}	1	*tetB+tetC+flor*	1
tetB+tetC	1	*rmtD+rmtB+* bla_{TEM-1}	1
tetC+flor	1	*tetB+tetC+flor+rmtB+sul1*	1

Table 6 QRDR mutations and antibiotic MIC values for 143 *H. parasuis* isolates.

QRDR mutation		Number of strains	MICs (μg/mL)					
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

Notes.

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.

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 6).

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 (Fig. 1).

DISCUSSION

In the current study, we observed high-level resistance to nalidixic acid and enrofloxacin. Similar results have been reported such as 84.8% to nalidixic acid (*Xu et al., 2011*) and 60.1% and 45.5% to enrofloxacin (*Xu et al., 2011*; *Zhang et al., 2013*). These differed from results in the United Kingdom and Spain (0 and 20%) (*De la Fuente et al., 2007*). We described the fluoroquinolone antimicrobial resistance profiles for *H. parasuis* strains isolated between 2014–2017. When compared with 2002–2009 and 2008-2010, our data indicated that fluoroquinolone antimicrobial resistance in *H. parasuis* was very serious in China during the last 15 years.

There have been few complete and systematic molecular studies of antimicrobial resistance in *H. parasuis*. The genes $bla_{\mathrm{ROB-1}}$, *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

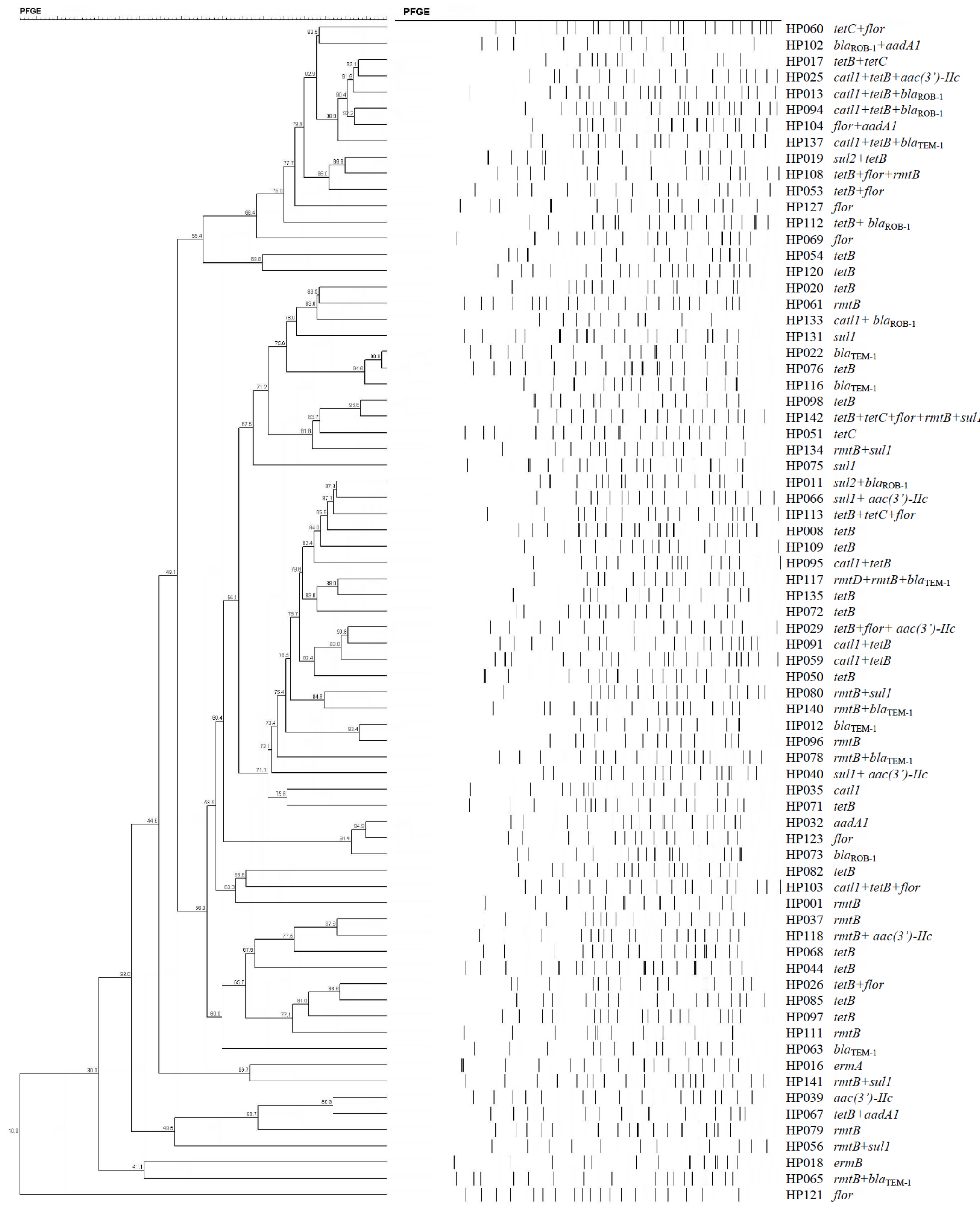

Figure 1 Dendrogram of patterns generated by PFGE of 73 ARG-containing *H. parasuis* isolates
Full-size DOI: 10.7717/peerj.4613/fig-1

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, the bla_{TEM-1} β-lactamase is the predominant genotype (Yang et al., 2017). In our study, we identified both bla_{TEM-1} and bla_{ROB-1} β-lactamase genes which are widespread among *H. parasuis* and *Pasteurella spp* (Guo et al., 2012; San Millan et al., 2007). bla_{TEM-1} and bla_{ROB-1} are usually present in *H. influenzae* and have particular geographic distributions in different countries (*Farrell et al., 2005*). These geographic differences may also be present in *H. parasuis*. The first reports of *bla*$_{TEM-1}$ and *bla*$_{ROB-1}$ were in China and Spain, respectively(*Guo et al., 2012*; *San Millan et al., 2007*). *bla*$_{ROB-1}$ was located on plasmid pB1000 and recently a novel 2,661 bp plasmid (pJMA-1) bearing *bla*$_{ROB-1}$ has been identified. This plasmid possessed a backbone found in small *Pasteurellaceae* plasmids and was 100% stable with a lower biological cost than pB1000 (*Moleres et al., 2015*).

We also identified genes encoding tetracycline efflux pumps (*tetB* and *tetD*) in this study. The first tetracycline resistant gene identified in *H. parasuis* was *tetB* and this gene is the most common tetracycline resistance gene in *Actinobacillus pleuropneumoniae* and *Pasturella multocida* (*Dayao et al., 2016*; *Matter et al., 2007*). The genes *tetH* and *tetM* are present in other members of the *Pasteurellaceae* (*Roberts, 2012*). Furthermore, the *tetB*-carrying plasmid pHS-Tet in *H. parasuis* was similar to a *tetL*-carrying plasmid in *Pasteurella* isolates (*Kehrenberg et al., 2005*; *Lancashire et al., 2005*). This is the first report of the *tetD* gene in *H. parasuis* isolates from China and needs further study. Tetracycline resistance genes are often associated with conjugative and mobile genetic elements enabling horizontal transfer (*Dayao et al., 2016*; *Roberts, 2012*). The presence of *tetD* suggests that tetracycline resistance in *H. parasuis* relies on efflux pumps.

In bacteria with animal origins, five florfenicol resistance genes (*floR, fexA, fexB, cfr* and *optrA*) have been reported (*Schwarz et al., 2004*; *Wang et al., 2015*). In Gram-negative bacteria, *floR* makes the greatest contribution to florfenicol resistance and this has been described for a number of bacterial species (*He et al., 2015*; *Meunier et al., 2010*; *Schwarz et al., 2004*; *Wang et al., 2015*). The emergence of florfenicol resistance in *H. parasuis* isolates was attributable to a novel small plasmid pHPSF1 bearing *floR*. This novel plasmid was similar to other *Pasteurellaceae* plasmids suggesting these species prefer to exchange genetic elements with each other.

High-level aminoglycoside resistance mediated by the production of the 16S rRNA methylases *armA, rmtA* to *H* and *npmA,* and resistance is increasing among Gram-negative pathogens (*Du et al., 2009*), being sometimes clonal spread of a single pulsotype (*Hopkins et al., 2010*). In our case, a clone bearing *rmtB* HP118 and HP037, was present in two different regions. However, until now, few studies have described the presence of the *armA* and *rmtB* genes in *H. parasuis* isolates, although they have been frequently reported on Enterobacteriaceae from food animals. The strains in our study also carried *rmtB, rmtD, aadA1* and *aac (3′) IIc* and these warrants further investigation.

The macrolide-resistance genes *erm A and erm B* showed a low frequency in our *H. parasuis* isolates. These genes are responsible for ribosomal binding site modifications that are the most important macrolide resistance mechanisms(*Takaya et al., 2010*).

The *sul1, sul2* and *sul3* genes are dihydropteroate synthases involved in sulfonamide resistance of Gram-negative bacteria and are usually associated with an integron system and a conjugative plasmid (*Vo et al., 2006*). In the current study, we identified both *sul1* and *sul2*, and these genes most likely accounted for the observed resistance to trimethoprim-sulfamethoxazole. These results are similar to others in Gram-negative bacteria (*Koljalg et al., 2009*; *Matter et al., 2007*).

This is the first report describing the presence of the *tetC, sul1, sul2, ermA, ermB, catl, rmtB, rmtD*, *aadA1* and *aac (3′)-IIc* genes in *H. parasuis,* to the best of our knowledge. Nevertheless, we did find several isolates with reduced antibiotic susceptibility that did not harbor any of the tested resistance genes. This suggests that *H. parasuis* possesses other resistance mechanisms such as mutations, decreases in permeability and increases in efflux pump activity or yet unknown antibiotic resistance mechanisms. In addition, the widespread dissemination of resistance genes and integrons could potentially fuel the rapid development of antimicrobial resistance due to their high transfer capabilities (Hussein et al., 2009). Therefore, more study is needed on this subject.

There have been numerous studies demonstrating *gyrA* and *parC* mutations engendering fluoroquinolone resistance in Gram-negative bacteria and Gram-positive bacteria from pigs such as *Salmonella spp*., *E. coli* or *Streptococcus suis* (Cao et al., 2017; Escudero et al., 2007). In *H. parasuis*, the *gyrA* mutations S83Y, S83F, D87Y, D87N and D87G are correlated with fluoroquinolone resistance. In addition, the *parC* mutations Y577C, V648I, E678D, S669F, A464V and A466S and *parE* mutations S283G, A227T and G241S were also found in these strains(Guo et al., 2011). In another study, mutations of *gyrA* D87N, *parC* S73R and *parE* T551A were involved in fluoroquinolone resistance, but other mutations such as in *gyrA* (452D^V/G, 627G^E), *gyrB* (211V^I, 254D^G), *parC* (73S^R/I, 227Q ∧H, 379L^I, 578C^Y) and *parE* (551T^A) occurred less frequently (Zhang et al., 2013). However, the *parE* mutation in *A. pleuropneumoniae* is possibly not involved in enrofloxacin resistance (Wang et al., 2010). In our study, most strains possessed *gyrA* mutations, and six strains possessed a *gyrA* mutation (D87H) not been previously reported. However, we do not know whether this mutation is directly related to fluoroquinolone resistance. We also identified four *parC* mutations. Unlike other studies, we found the *parC* 578 mutation in both resistant and sensitive strains, suggesting this mutation is not involved in resistance (Zhang et al., 2013). Overall, the QRDR analysis in our study suggested that the mutations at codon 83 or 87 of *gyrA* were responsible for fluoroquinolone resistance and that *gyrB* and *parE* were not.

Interestingly, our PFGE results indicated that almost 70% of our *H. parasuis* were genetically diverse, similar to a recent report (Guo et al., 2012). These results are in contrast to a previous study presenting evidence for the clonal spread of β-lactam resistance (San Millan et al., 2007). Our data suggests that resistance genes are spread *via* transferable elements such as plasmids and transposons in addition to clonal spread. Therefore, research on mechanisms for the spread of antimicrobial resistance in *H. parasuis* needs further investigation.

CONCLUSIONS

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

ACKNOWLEDGEMENTS

We thank members of our laboratories for fruitful discussions.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the National Natural Science Foundation of China (No. 31372479), Science and Technology Planning Project of Guangdong Province, China (No. 2015B090901059), and the National Key Research Program of China (grant 2017YFD0501404). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31372479.
Science and Technology Planning Project of Guangdong Province: 2015B090901059.
National Key Research Program of China: 2017YFD0501404.

Competing Interests

Lili Guo is an employee of Qingdao Yebio Biological Engineering Co., Ltd.

Author Contributions

- Yongda Zhao conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Lili Guo performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jie Li performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Xianhui Huang contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Binghu Fang conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The animal research committees of the South China Agriculture University granted approval for this research.

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in a Supplemental Information 1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4613#supplemental-information.

REFERENCES

Aarestrup FM, Seyfarth AM, Angen O. 2004. Antimicrobial susceptibility of *Haemophilus parasuis* and Histophilus somni from pigs and cattle in Denmark. *Veterinary Microbiology* **101**:143–146 DOI 10.1016/j.vetmic.2004.02.012.

Angen O, Oliveira S, Ahrens P, Svensmark B, Leser TD. 2007. Development of an improved species specific PCR test for detection of *Haemophilus parasuis*. *Veterinary Microbiology* **119**:266–276 DOI 10.1016/j.vetmic.2006.10.008.

Cao TT, Deng GH, Fang LX, Yang RS, Sun J, Liu YH, Liao XP. 2017. Characterization of Quinolone Resistance in *Salmonella enterica* from Farm Animals in China. *Journal of Food Protection* **80**:1742–1748 DOI 10.4315/0362-028X.JFP-17-068.

Cavaco LM, Hasman H, Xia S, Aarestrup FM. 2009. qnrD, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrobial Agents and Chemotherapy* **53**:603–608 DOI 10.1128/AAC.00997-08.

Clinical and Laboratory Standards Institute (CLSI). 2015. *Performance standards for antimicrobial disk and dilution susceptibility tests for Haemophilus influenzae and Haemophilus parainfluenzae*. Wayne: Clinical and Laboratory Standards Institute, M02 & M07.

Dayao DAE, Gibson JS, Blackall PJ, Turni C. 2016. Antimicrobial resistance genes in *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Pasteurella* multocida isolated from Australian pigs. *Australian Veterinary Journal* **94**:227–231 DOI 10.1111/avj.12458.

De Gheldre Y, Avesani V, Berhin C, Delmee M, Glupczynski Y. 2003. Evaluation of Oxoid combination discs for detection of extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy* **52**:591–597 DOI 10.1093/jac/dkg415.

De la Fuente AJ, Tucker AW, Navas J, Blanco M, Morris SJ, Gutierrez-Martin CB. 2007. Antimicrobial susceptibility patterns of *Haemophilus parasuis* from pigs in the United Kingdom and Spain. *Veterinary Microbiology* **120**:184–191 DOI 10.1016/j.vetmic.2006.10.014.

Doi Y, Arakawa Y. 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clinical Infectious Diseases* **45**:88–94 DOI 10.1086/518605.

Du XD, Wu CM, Liu HB, Li XS, Beier RC, Xiao F, Qin SS, Huang SY, Shen JZ. 2009. Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens. *Journal of Antimicrobial Chemotherapy* **64**:1328–1330 DOI 10.1093/jac/dkp354.

Escudero JA, San Millan A, Catalan A, De la Campa AG, Rivero E, Lopez G, Dominguez L, Moreno MA, Gonzalez-Zorn B. 2007. First characterization of fluoroquinolone resistance in *Streptococcus suis*. *Antimicrobial Agents and Chemotherapy* **51**:777–782 DOI 10.1128/AAC.00972-06.

Farrell DJ, Morrissey I, Bakker S, Buckridge S, Felmingham D. 2005. Global distribution of TEM-1 and ROB-1 beta-lactamases in Haemophilus influenzae. *Journal of Antimicrobial Chemotherapy* **56**:773–776 DOI 10.1093/jac/dki281.

Grobner S, Linke D, Schutz W, Fladerer C, Madlung J, Autenrieth IB, Witte W, Pfeifer Y. 2009. Emergence of carbapenem-non-susceptible extended-spectrum beta-lactamase-producing Klebsiella pneumoniae isolates at the university hospital of Tubingen, Germany. *Journal of Medical Microbiology* **58**:912–922 DOI 10.1099/jmm.0.005850-0.

Guo LL, Zhang JM, Xu CG, Ren T, Zhang B, Chen JD, Liao M. 2012. Detection and characterization of beta-lactam resistance in *Haemophilus parasuis* strains from pigs in South China. *Journal of Integrative Agriculture* **11**:116–121 DOI 10.1016/S1671-2927(12)60789-5.

Guo LL, Zhang JM, Xu CG, Zhao YD, Ren T, Zhang B, Fan HY, Liao M. 2011. Molecular characterization of fluoroquinolone resistance in *Haemophilus parasuis* isolated from pigs in South China. *Journal of Antimicrobial Chemotherapy* **66**:539–542 DOI 10.1093/jac/dkq497.

He T, Shen JZ, Schwarz S, Wu CM, Wang Y. 2015. Characterization of a genomic island in Stenotrophomonas maltophilia that carries a novel floR gene variant. *Journal of Antimicrobial Chemotherapy* **70**:1031–1036 DOI 10.1093/jac/dku491.

Hopkins KL, Escudero JA, Hidalgo L, Gonzalez-Zorn B. 2010. 16S rRNA methyltransferase RmtC in *Salmonella enterica* serovar Virchow. *Emerging Infectious Diseases* **16**:712–715 DOI 10.3201/eid1604.090736.

Hou J, Yang X, Zeng Z, Lv L, Yang T, Lin D, Liu JH. 2013. Detection of the plasmid-encoded fosfomycin resistance gene fosA3 in *Escherichia coli* of food-animal origin. *Journal of Antimicrobial Chemotherapy* **68**:766–770 DOI 10.1093/jac/dks465.

Hussein AI, Ahmed AM, Sato M, Shimamoto T. 2009. Characterization of integrons and antimicrobial resistance genes in clinical isolates of Gram-negative bacteria from Palestinian hospitals. *Microbiology and Immunology* **53**:595–602 DOI 10.1111/j.1348-0421.2009.00168.x.

Kehrenberg C, Catry B, Haesebrouck F, De Kruif A, Schwarz S. 2005. tet(L)-mediated tetracycline resistance in bovine Mannheimia and *Pasteurella* isolates. *Journal of Antimicrobial Chemotherapy* **56**:403–406 DOI 10.1093/jac/dki210.

Koljalg S, Truusalu K, Vainumae I, Stsepetova J, Sepp E, Mikelsaar M. 2009. Persistence of *Escherichia coli* clones and phenotypic and genotypic antibiotic resistance in recurrent urinary tract infections in childhood. *Journal of Clinical Microbiology* **47**:99–105 DOI 10.1128/JCM.01419-08.

Lancashire JF, Terry TD, Blackall PJ, Jennings MP. 2005. Plasmid-encoded Tet B tetracycline resistance in *Haemophilus parasuis*. *Antimicrobial Agents and Chemotherapy* **49**:1927–1931 DOI 10.1128/AAC.49.5.1927-1931.2005.

Li B, Zhang Y, Wei J, Shao D, Liu K, Shi Y, Qiu Y, Ma Z. 2015. Characterization of a novel small plasmid carrying the florfenicol resistance gene floR in *Haemophilus parasuis*. *Journal of Antimicrobial Chemotherapy* **70**:3159–3161 DOI 10.1093/jac/dkv230.

Liu JH, Wei SY, Ma JY, Zeng ZL, Lu DH, Yang GX, Chen ZL. 2007. Detection and characterisation of CTX-M and CMY-2 beta-lactamases among *Escherichia coli* isolates from farm animals in Guangdong Province of China. *International Journal of Antimicrobial Agents* **29**:576–581 DOI 10.1016/j.ijantimicag.2006.12.015.

Maka L, Popowska M. 2016. Antimicrobial resistance of *Salmonella spp.* isolated from food. *Roczniki Państwowego Zakładu Higieny* **67**:343–358.

Markowska-Daniel I, Urbaniak K, Stepniewska K, Pejsak Z. 2010. Antibiotic susceptibility of bacteria isolated from respiratory tract of pigs in Poland between 2004 and 2008. *Polish Journal of Veterinary Sciences* **13**:29–36.

Matter D, Rossano A, Limat S, Vorlet-Fawer L, Brodard I, Perreten V. 2007. Antimicrobial resistance profile of *Actinobacillus pleuropneumoniae* and Actinobacillus porcitonsillarum. *Veterinary Microbiology* **122**:146–156 DOI 10.1016/j.vetmic.2007.01.009.

Meunier D, Jouy E, Lazizzera C, Doublet B, Kobisch M, Cloeckaert A, Madec JY. 2010. Plasmid-borne florfenicol and ceftiofur resistance encoded by the floR and blaCMY-2 genes in *Escherichia coli* isolates from diseased cattle in France. *Journal of Medical Microbiology* **59**:467–471 DOI 10.1099/jmm.0.016162-0.

Miranda JM, Rodriguez JA, Galan-Vidal CA. 2009. Simultaneous determination of tetracyclines in poultry muscle by capillary zone electrophoresis. *Journal of Chromatography A* **1216**:3366–3371 DOI 10.1016/j.chroma.2009.01.105.

Moleres J, Santos-Lopez A, Lazaro I, Labairu J, Prat C, Ardanuy C, Gonzalez-Zorn B, Aragon V, Garmendia J. 2015. Novel blaROB-1-bearing plasmid conferring resistance to beta-lactams in *Haemophilus parasuis* isolates from healthy weaning pigs. *Applied and Environmental Microbiology* **81**:3255–3267 DOI 10.1128/AEM.03865-14.

Oliveira S, Pijoan C. 2004. *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Veterinary Microbiology* **99**:1–12 DOI 10.1016/j.vetmic.2003.12.001.

Roberts MC. 2012. Acquired tetracycline resistance genes. In: *Antimicrobial discovery and development*. Boston: Springer.

Sambrook J, Russell D. 2001. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

San Millan A, Escudero JA, Catalan A, Nieto S, Farelo F, Gibert M, Moreno MA, Dominguez L, Gonzalez-Zorn B. 2007. Beta-lactam resistance in *Haemophilus parasuis* Is mediated by plasmid pB1000 bearing blaROB-1. *Antimicrobial Agents and Chemotherapy* **51**:2260–2264 DOI 10.1128/AAC.00242-07.

San Millan A, Escudero JA, Gifford DR, Mazel D, MacLean RC. 2016. Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria. *Nature Ecology & Evolution* **1(1)**:10 DOI 10.1038/s41559-016-0010.

Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A. 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *Fems Microbiology Reviews* **28**:519–542 DOI 10.1016/j.femsre.2004.04.001.

Shibata N, Doi Y, Yamane K, Yagi T, Kurokawa H, Shibayama K, Kato H, Kai K, Arakawa Y. 2003. PCR typing of genetic determinants for metallo-beta-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *Journal of Clinical Microbiology* **41**:5407–5413 DOI 10.1128/JCM.41.12.5407-5413.2003.

Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. 1996. Detection of erythromycin-resistant determinants by PCR. *Antimicrobial Agents and Chemotherapy* **40**:2562–2566.

Takaya A, Kitagawa N, Kuroe Y, Endo K, Okazaki M, Yokoyama E, Wada A, Yamamoto T. 2010. Mutational analysis of reduced telithromycin susceptibility of Streptococcus pneumoniae isolated clinically in Japan. *FEMS Microbiology Letters* **307**:87–93 DOI 10.1111/j.1574-6968.2010.01962.x.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**:2233–2239.

Vo ATT, Van Duijkeren E, Fluit AC, Wannet WJB, Verbruggen AJ, Maas HME, Gaastra W. 2006. Antibiotic resistance, integrons and Salmonella genomic island 1 among non-typhoidal Salmonella serovars in The Netherlands. *International Journal of Antimicrobial Agents* **28**:172–179 DOI 10.1016/j.ijantimicag.2006.05.027.

Walsh C, Fanning S. 2008. Antimicrobial resistance in foodborne pathogens—a cause for concern? *Current Drug Targets* **9**:808–815 DOI 10.2174/138945008785747761.

Wang J, Lin DC, Guo XM, Wei HK, Liu XQ, Chen XJ, Guo JY, Zeng ZL, Liu JH. 2015. Distribution of the multidrug resistance gene cfr in Staphylococcus isolates from pigs, workers, and the environment of a hog market and a slaughterhouse in Guangzhou, China. *Foodborne Pathogens and Disease* **12**:598–605 DOI 10.1089/fpd.2014.1891.

Wang YC, Chan JP, Yeh KS, Chang CC, Hsuan SL, Hsieh YM, Chang YC, Lai TC, Lin WH, Chen TH. 2010. Molecular characterization of enrofloxacin resistant *Actinobacillus pleuropneumoniae* isolates. *Veterinary Microbiology* **142**:309–312 DOI 10.1016/j.vetmic.2009.09.067.

Weill FX, Demartin M, Tande D, Espie E, Rakotoarivony I, Grimont PA. 2004. SHV-12-like extended-spectrum-beta-lactamase-producing strains of *Salmonella enterica* serotypes Babelsberg and Enteritidis isolated in France among infants adopted from Mali. *Journal of Clinical Microbiology* **42**:2432–2437 DOI 10.1128/JCM.42.6.2432-2437.2004.

Wissing A, Nicolet J, Boerlin P. 2001. The current antimicrobial resistance situation in Swiss veterinary medicine. *Schweiz Arch Tierheilkd* **143**:503–510.

Xu C, Zhang J, Zhao Z, Guo L, Zhang B, Feng S, Zhang L, Liao M. 2011. Antimicrobial susceptibility and PFGE genotyping of *Haemophilus parasuis* isolates from pigs in

South China (2008–2010). *Journal of Veterinary Medical Science* **73**:1061–1065 DOI 10.1292/jvms.10-0515.

Xu R, Yang ZH, Wang QP, Bai Y, Liu JB, Zheng Y, Zhang YR, Xiong WP, Ahmad K, Fan CZ. 2018. Rapid startup of thermophilic anaerobic digester to remove tetracycline and sulfonamides resistance genes from sewage sludge. *Science of the Total Environment* **612**:788–798 DOI 10.1016/j.scitotenv.2017.08.295.

Yang Y, Chen J, Lin D, Xu X, Cheng J, Sun C. 2017. Prevalence and drug resistance characteristics of carbapenem-resistant Enterobacteriaceae in Hangzhou, China. *Frontiers in Medicine* Epub ahead of print July 8 2017.

Zhang J, Xu C, Guo L, Ke B, Ke C, Zhang B, Deng XL, Liao M. 2011. A rapid pulsed-field gel electrophoresis method of genotyping *Haemophilus parasuis* isolates. *Letters in Applied Microbiology* **52**:589–595 DOI 10.1111/j.1472-765X.2011.03048.x.

Zhang J, Xu C, Shen H, Li J, Guo L, Cao G, Feng S, Liao M. 2014. Biofilm formation in *Haemophilus parasuis*: relationship with antibiotic resistance, serotype and genetic typing. *Research in Veterinary Science* **97**:171–175 DOI 10.1016/j.rvsc.2014.04.014.

Zhang Q, Zhou M, Song D, Zhao J, Zhang A, Jin M. 2013. Molecular characterisation of resistance to fluoroquinolones in *Haemophilus parasuis* isolated from China. *International Journal of Antimicrobial Agents* **42**:87–89 DOI 10.1016/j.ijantimicag.2013.03.011.

Zhao J, Chen Z, Chen S, Deng Y, Liu Y, Tian W, Huang X, Wu C, Sun Y, Sun Y, Zeng Z, Liu JH. 2010. Prevalence and dissemination of oqxAB in *Escherichia coli* isolates from animals, farmworkers, and the environment. *Antimicrobial Agents and Chemotherapy* **54**:4219–4224 DOI 10.1128/AAC.00139-10.

Zhou XL, Xu XJ, Zhao YX, Chen P, Zhang X, Chen HC, Cai XW. 2010. Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates. *Veterinary Microbiology* **141**:168–173 DOI 10.1016/j.vetmic.2009.05.012.