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Laboratory Detection of *Haemophilus parasuis* with Decreased Susceptibility to Nalidixic Acid and Enrofloxacin Due to GyrA and ParC Mutations

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Abstract: The detection of pathogens with decreased susceptibility to antibacterial and elucidating the molecular mechanism of resistance are of epidemiological and clinical interest in animal husbandry. The 21 *Haemophilus parasuis* clinical isolates and one American Type Culture Collection reference strains of *Actinobacillus pleuropneumoniae* (ATCC 27090) were screened for susceptibility to nalidixic acid and enrofloxacin by the microdilution methods. The nalidixic acid MICs for all of the isolates were ≥ 2 μg mL^{-1} and the enrofloxacin MICs for isolates were ≤ 8 μg mL^{-1}. In addition, the Quinolone Resistance-Determining Regions (QRDRs) of gyrA and parC of all strains were sequenced. Strains for which nalidixic acid MICs were ≤ 4.0 μg mL^{-1} lacked modifications in the QRDRs of GyrA and ParC. In contrast, all strains for which nalidixic acid MICs were ≥ 8 μg mL^{-1} exhibited one or more amino acid changes in GyrA and ParC. Mutations in GyrA is the major resistance mechanism of *Haemophilus parasuis* to nalidixic acid and enrofloxacin.

Key words: Detection, enrofloxacin, resistance, collection, nalidixic acid, China

INTRODUCTION

Haemophilus parasuis is a common bacteria of the upper respiratory tract of pigs which is the causative agent of septicaemia without polyserositis and Glasser's disease characterized by fibrinous polyserositis, arthritis and meningitis (Nedbalcova *et al*., 2006). Although, nutrition, sanitation, management and vaccination (Commercial or autogenous vaccines) are useful in preventing *Haemophilus parasuis* infections on pig farms, oral or parenteral administration of high dose of antibacterial drug is necessary. However, the application of high dose of antibacterial drug for prophylactic or treatment of Glasser's disease may lead to selective drug resistant strains. High frequencies of resistance to multiple antibacterial drug including Fluoro Quinolone (FQ) has been reported in several countries to date (Aarestrup *et al*., 2004; De la Fuente *et al*., 2007; San *et al*., 2007; Zhou *et al*., 2010). The reports from China also indicated that the prevalence of Enrofloxacin (ER) resistant *Haemophilus parasuis* was popular (Zhou *et al*., 2010). Many fluoroquinolone antibacterial drug has been approved for used in swine industry in china such as norfloxacin, ciprofloxacin, enrofloxacin, sarafloxacin and danofloxacin. While this kind of substance has not been approved for used in animal husbandry in Australia and USA. Fluoroquinolones can bind to gyrase or topoisomerase IV in the presence of DNA, then alter protein conformation and finally inhibit bacterial DNA replication. The major mechanism of fluoroquinolone resistance is mutations of the Quinolone Resistance-Determining Regions (QRDRs) in gyrase (encoded by the gyrA and gyrB genes) and topoisomerase IV (encoded by parC and parE) (Drlica, 1999). However, molecular mechanisms of decreased susceptibility to fluoroquinolones in *Haemophilus parasuis* has not been elucidated up to now.

The goal of this study is to detect the susceptibility of *Haemophilus parasuis* to nalidixic acid and enrofloxacin and elucidate the association of amino acid changes in the QRDRs of GyrA and ParC and decreased susceptibilities to nalidixic acid and enrofloxacin.

MATERIALS AND METHODS

Bacterial strains: About 21 *Haemophilus parasuis* isolates were obtained from the lung of pigs in different herds located in central China between September 2009

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Table 1: Oligonucleotide primers used in this study

Genes	Primers	Sequence (5'-3')	Product size	Gene fragment[a]
16sRNA	16sRNA F	GTG ATG AGG AAG GGT GGT GT	821	394-413
	16sRNA R	GGC TTC GTC ACC CTC TGT		1198-1215
gyrA	gyrA F	GCCCGTTCATCGACGCGTACT	538	126-146
	gyrA R	TCGCCGCCGTTGGGAAATCA		645-664
parC	parC F	TTT ATT GGC GAC GGT CTA	796	76-93
	parC R	ACA ATG CGG ATA GGG TTT		855-872

[a]Nucleotide positions are based on *Hæmophilus parasuis* 0165 gene sequences

and July 2010. The isolates were identified by PCR assays (Oliveira *et al*., 2001) with the primer shown in Table 1 and serotyped rapidly by slide agglutination tests (Nielsen, 1993). No genetic relationship was observed among the isolates by ERIC-PCR analysis. All isolates were stored in 10% skim milk at -80°C until use.

Antimicrobial susceptibility testing: The MIC values of nalidixic acid and enrofloxacin (China Institute of Veterinary Drug Control) were determined in veterinary fastidious medium by microdilution susceptibility test following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) document M_{31}-A_2 (CLSI, Document M31-A3, 2008). *Actinobacillus pleuropneumoniae* ATCC 27090 was used as a quality control for antimicrobial susceptibility test.

Sequence analysis of the QRDRs: To determine the QRDRs mutations in *Haemophilus parasuis*, the 21 isolates were analysed for gyrA and parC sequences using the primers shown in Table 1. The primers were designed based on the sequences of *Haemophilus parasuis gyrA* and *parC* genes (GenBank accession number NC_011852). PCR amplified products were purified with the Qiagen quick PCR Purification Kit following the manufacturer's recommendations and send to Sangon Biotech (Shanghai) Co., Ltd for sequencing.

RESULTS AND DISCUSSION

Nalidixic acid and enrofloxacin susceptibility in *Haemophilus parasuis*: The MIC values of nalidixic acid and enrofloxacin were shown in Table 2. Compared with other studies, different fluoroquinolone susceptibilities profile of *Haemophilus parasuis* have been observed in different countries (Aarestrup *et al*., 2004; De la Fuente *et al*., 2007). Although, no approved interpretive criteria resistance breakpoint are currently available to assess nalidixic acid and enrofloxacin susceptibility or resistance of *Haemophilus parasuis*, isolates which exhibit higher minimum inhibitory concentrations are assumed to possess resistance mechanisms.

Mutations in QRDRs of *Haemophilus parasuis* isolates: GyrA and parC mutations were identified by sequencing (Table 2). Overall, 5 different substitutions in GyrA

Table 2: Amino acid substitutions in the QRDRs of GyrA and ParC of the *Hæmophilus parasuis* strains

Number of strains	MIC ($\mu g\ mL^{-1}$) Nalidixic acid	Enrofloxacin	Amino acid change (Codon change)* GyrA 83	87	ParC 73	77
Type strain	1	<0.125	S	D	S	E
7	16->32	0.5-2.0	F	N	R	-
4	>32	0.5-1	F	N	I	-
1	>32	8	F	N	I	G
1	>32	1	F	N	I	K
3	>32	1	F	N	S	K
1	8	<0.125	F	-	-	-
1	>32	1	F	G	-	K
2	>32	0.5-1	F	Y	I	-
1	>32	0.125	Y	-	R	-

*:D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; N, asparagine; R, arginine; S, serine; Y, tyrosine; -, wild type

(S83F, S83Y, D87G, D87N and D87Y), 4 different substitutions in ParC (S73I, S73R, E77G and E77 K) were discovered in the resistant strain. Similar to *Haemophilus influenzae* (Perez-Vazquez *et al*., 2004), resistant to quinolone in *Haemophilus parasuis* is associated with mutations in both gyrA and parC. Isolates with the nalidixic aid MIC $\geq 8\ \mu g\ mL^{-1}$ carried at least one mutation in the gyrA, leading to amino acid changes in codon 83 and/or 87. The enrofloaxacin MIC values of 19 isolates with double mutations in codon 83 and 87 of gyrA were detected as 0.5-8 $\mu g\ mL^{-1}$.

In the present study, several QRDR mutations profile were found among decrease susceptibility strains (Table 2). It is worth noting that at least one mutation in GyrA was present in all strains and 90% of isolates had two mutations in GyrA. Th result suggests that GyrA mutations (S83F, S83Y, D87G, D87N and D87Y) are closely correlated with nalidixic acid and enrofloxacin resistance in *Haemophilus parasuis.* The fluoroquinolone resistance levels of the strains with double GyrA mutations were higher than those with a single GyrA mutation indicating that the increasing MICs of fluoroquinolones for *Haemophilus parasuis* are also associated with stepwise accumulation of GyrA mutations. The results are agreement with the data of (Nair *et al*., 2006) who demonstrated that a single GyrA mutation at Ser-83 alone resulted in resistance to nalidixic acid and

reduced susceptibility to ciprofloxacin (MICs of 0.125-0.25 μg mL^{-1}). Therefore, it was concluded that Ser-83 is an important site for determining fluoroquinolone resistance within *Haemophilus parasuis* isolate.

Increasing nalidixic acid MIC values correlating with stepwise accumulation of mutations in the GyrA suggests that DNA gyrase is a primary target of nalidixic acid in *Haemophilus parasuis* as in other gram negative bacteria. Strains with different ParC mutations (S73I, S73R, E77G and E77K) profile display similar susceptibility to nalidixic aicd and enrofloxacin except one isolate, indicating that these mutations are possibly not directly related to fluoroquinolone resistance of *Haemophilus parasuis.* One strain with the same mutation in GyrA with other strain but the mutations involved in substitutions of Ser73Ile and Gln77Gly of ParC. The MIC value to enrofloxacin of this strain is 8 μg mL^{-1} suggested that the mutation profile in ParC may change the susceptibility or the strain posses additional resistance mechanisms such as additional mutation in the drug target GyrB and ParE, over expression of an efflux pump system and/or decreased permeation of the drug. The possibility of those has not been examined in the strain. Further studies are necessary to evaluate the contribution of GyrB, ParC and ParE mutations to enrofloxacin resistance in *Haemophilus parasuis*.

Many previous studies have demonstrated that fluoroquinolones resistance mutations occur first in GyrA in gram negative bacteria but they occur first in ParC in gram positive bacteria. Topoisomerase IV is the secondary target for fluoroquinolones and a ParC mutation related to fluoroquinolone resistance in other gram negative bacteria such as Salmonella and *E. coli*.

Nalidixic acid has been received special attention for use in a screening test for determination of the susceptibilities of gram negative bacteria to quinolones (Crump *et al*., 2003; Hakanen *et al*., 1999). In this study, all strain with high MIC to nalidixic acid were less sensitive to enrofloxacin and mutation only occur in the strain with MIC >8 μg mL^{-1}. The result suggested that sensitivity to nalidixic acid can be used to screen for sensitivity to fluoquinolone antibacterial drug in *Haemophilus parasuis*.

Respiratory system disease is the major disease in china pig industry. Antibacterial agents such as enrofloxacin and florfenicol has been used as preventive drug widely. With no resistance breakpoint for *Haemophilus parasuis* susceptibility test, the screening proposal criteria are intended to identify isolates with a resistance mechanism (mutations in the QRDR of GyrA) that may be responsible for the spread of quinolone resistance.

Plasmid mediated other antibacterial drug resistance have been found in *Haemophilus parasuis* (Lancashire *et al*., 2005; San *et al*., 2007). But plasmid mediated quinolone resistance determinants were not found in *Haemophilus parasuis* isolates. Further investigation is needed to identify if plasmid mediated quinolone resistance determinants exist in *Haemophilus parasuis*. More extensive surveillance might reveal the status of quinolone-resistant strains and will help clarify the epidemiological relationships in fluoroquinolone-resistant *Haemophilus parasuis.*

CONCLUSION

This is the first report focused on fluoroquinolone resistance mechanisms in *Haemophilus parasuis* isolates. Nalidixic acid and enrofloxacin resistance of *Haemophilus parasuis* appears to be linked to multiple target gene mutations at codon positions 83 and 87 of gyrA and codon positions 73 and 77 of parC.

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