Veterinary Microbiology 142 (2010) 309–312

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Molecular characterization of enrofloxacin resistant *Actinobacillus pleuropneumoniae* isolates

Yu-Chih Wang [a], Jacky Peng-Wen Chan [a], Kuang-Sheng Yeh [c], Chao-Chin Chang [b], Shih-Ling Hsuan [d], Yi-Mei Hsieh [b], Yi-Chih Chang [a,e], Tan-Chen Lai [a,f], Wen-Hsin Lin [g], Ter-Hsin Chen [b,d,*]

[a] *Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan*
[b] *The Graduate Institute of Veterinary Public Health, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan*
[c] *Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[d] *Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan*
[e] *Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan*
[f] *Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung, Taiwan*
[g] *School of Pharmacy, China Medical University, Taichung, Taiwan*

ARTICLE INFO

Article history:
Received 3 March 2009
Received in revised form 14 September 2009
Accepted 16 September 2009

Keywords:
Enrofloxacin resistance
Actinobacillus pleuropneumoniae
Quinolone resistance-determining region
Efflux pump

ABSTRACT

Enrofloxacin (ER) resistant *Actinobacillus pleuropneumoniae* strains emerged in Taiwan in 2002. The mechanism of ER resistance in *A. pleuropneumoniae* has not yet been reported. A total of 48 *A. pleuropneumoniae* isolates were obtained from the lungs of pigs with pleuropneumonia in Taiwan between September 2007 and April 2008. Twenty-nine isolates were found to be resistant to enrofloxacin. To understand the mechanisms of *A. pleuropneumoniae*'s resistance to ER, enrofloxacin susceptibility of the isolates along with the mutations of the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, *qnr* genes were analyzed. Enrofloxacin resistant isolates were found to carry at least one mutation in the QRDR of *gyrA*, leading to amino acid changes at codon 83 or 87. Efflux pump inhibitor (Phe-Arg-β-naphthylamide) decreased enrofloxacin minimum inhibitory concentration 2–16-fold, suggesting participation of efflux in ER resistance. Plasmid mediated quinolone resistance genes *qnr* were not detected in these isolates. In conclusion, enrofloxacin resistance of *A. pleuropneumoniae* may be linked to multiple target gene mutations and active effluxs.

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1. Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious economically important endemic swine disease worldwide (Bosse et al., 2002). Although management, sanitation, nutrition, and vaccination are useful in preventing *A. pleuropneumoniae* infections on pig farms, use of antibiotics for treatment of infected pigs is necessary in avoiding economic loss. However, the application of antibiotics in the treatment of pig's pleuropneumonia may lead to selective survival of drug resistant strains. A rise in frequencies of resistance to multiple antibiotics has been reported in several countries to date (Gutiérrez-Martín et al., 2006; Hendriksen et al., 2008). Previous reports indicated that fluoroquinolone (FQ) resistant *A. pleuropneumoniae* isolates were found in Taiwan, Denmark, Poland and England (Chang et al., 2002; Hendriksen et al., 2008). The reports from Taiwan also indicated that the prevalence of enrofloxacin (ER) resistant *A. pleuropneumoniae* was high (Chang et al., 2002). However, the mechanism for the acquisition of ER resistance in *A. pleuropneumoniae* has not yet been elucidated.

* Corresponding author at: 250, Kuo Kuang Rd., Taichung 402, Taiwan. Tel.: +886 4 22840894x703; fax: +886 4 22852186.
E-mail address: thc@dragon.nchu.edu.tw (T.-H. Chen).

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doi:10.1016/j.vetmic.2009.09.067

In Gram negative bacteria, FQ resistance occurs mainly by interplay of three mechanisms. This is realized by stepwise accumulation of mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, active efflux of FQs and the presence of the plasmid-borne resistance genes (*qnr*) protecting the target topoisomerase (Cárdenas et al., 2001; Chu et al., 2005; Gay et al., 2006). Herein, we report the nucleotide sequence of the *gyrA*, *gyrB*, *parC*, and *parE* of the ER resistant *A. pleuropneumoniae*, and the presence of *gnr* gene in these isolates. Although no approved interpretive criteria are currently available to assess enrofloxacin susceptibility or resistance of *A. pleuropneumoniae*, isolates which exhibit higher minimum inhibitory concentrations are assumed to possess resistance mechanisms. In this study, we report the nucleotide sequences of the target genes *gyrA*, *gyrB*, *parC* and *parE* of such *A. pleuropneumoniae* isolates. In order to assess whether other resistance mechanisms might contribute to the observed elevated minimum inhibitory concentration (MIC) of enrofloxacin, the corresponding *A. pleuropneumoniae* isolates have also been investigated for the presence of transferable quinolone resistance genes by PCR and the involvement of active efflux by comparatively determining MICs in the presence and absence of the efflux pump inhibitor Phe-Arg-β-naphthylamide.

2. Materials and methods

2.1. Bacterial strains

A total of 196 pigs that died of pleuropneumonia from 35 herds located in central Taiwan between September 2007 and April 2008 were sampled. Forty-eight *A. pleuropneumoniae* isolates were obtained from the lungs of pigs in 20 different herds. The isolates were serotyped by PCR assays (Gram et al., 2000; Jessing et al., 2003; Schuchert et al., 2004) and rapid slide agglutination tests (Nielsen, 1986).

2.2. Antimicrobial susceptibility testing

Susceptibility of florfenicol and ceftiofur was determined by disc diffusion test following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) M31-A2. *A. pleuropneumoniae* ATCC 27090 was used as a quality control isolate for antimicrobial susceptibility test. The MIC values of ER were determined in Veterinary Fastidious Medium by broth microdilution method. MICs in the presence of 80 μg/ml of the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) were also tested.

2.3. Sequence analysis of the QRDR

To identify the QRDR mutations in the ER resistant isolates, the *gyrA*, *gyrB*, *parC* and *parE* genes were amplified and sequenced using primers listed in Table 1. These primers were constructed using *A. pleuropneumoniae* L20 and JL03 gene sequence data (GenBank accession number CP000569 and CP000687). The PCR was performed in 50-μl volumes consisting of a 200 μM of dNTP, 1.5 mM $MgCl_2$, 1 U of gold *Taq* DNA polymerase, and 50 pmol of each primer. The PCR was carried out using a TaKaRa PCR Thermal Cycler Dice with an initial denaturing cycle at 95 °C for 3 min; followed by 35 cycles at 95 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min; with a final extension step at 72 °C for 7 min. PCR amplified products were purified with the QIAquick PCR Purification Kit, following the manufacturer's recommendations for sequencing. All the sequencing was performed with ABI prism 3700 Automated DNA Analyzer (Perkin-Elmer Applied Biosystems).

2.4. PCR for the detection of qnr genes

PCR was used to simultaneously detect *qnrA*, *qnrB*, *qnrS* and *qepA* genes using specific primers previously reported for *Klebsiella pneumoniae* or *Escherichia coli* (Wu et al., 2008a,b; Yamane et al., 2007).

2.5. Nucleotide sequence accession number

The nucleotide sequences of the variant *gyrA*, *parC* and *parE* genes reported here have been submitted to the GenBank database under the accession numbers GQ360039–GQ360058.

3. Results

3.1. A. pleuropneumoniae serotyping

A total of 48 *A. pleuropneumoniae* isolates were serotyped. Among the five serotypes detected, serovar 1 was predominant (41 isolates), followed by serovar 5 (3 isolates), serovar 2 (2 isolates), serovar 7(1 isolate) and serovar 8 (1 isolate). The incidence of the serotype of the isolates in this study was consistent with the pattern previously reported in Taiwan (Chang et al., 2002). Serovar 1 was also the most commonly isolated *A. pleuropneumoniae* from pigs in Taiwan.

Table 1
Oligonucleotide primers used in this study.

Gene	Primer		Product size	Gene fragment[a]
gyrA	APgyrAF	5′-ATGAGCGAATTAGCCAAAGA	582 bp	1–20
	APgyrAR	5′-GCAACCGTCCAACACTTCAT		563–582
gyrB	APgyrBF	5′-ACCGCCCGCAGAGTCACCC	458 bp	1293–1311
	APgyrBR	5′-CGCACTTAGCCGGTTTCCG		854–872
parC	APparCF	5′-TGCCGTTTATCGGTGACG	567 bp	71–88
	APparCR	5′-CTGCCTCAGTCGGGTAAT		620–637
parE	APparEF	5′-TCTCATCGGCGCAAAGTCGT	408 bp	1121–1140
	APparER	5′-GGCGCAAGAACAAGGCACAT		1509–1528

[a] Nucleotide positions are based on *Actinobacillus pleuropneumoniae* L20 and JL03 gene sequences.

Table 2
Susceptibility to enrofloxacin and topoisomerase mutations.

N	Serotype (*n*)	Enrofloxacin MIC	Enrofloxacin MIC with PAβN[a]	Farm (*n*)	Mutation in the QRDR		
					gyrA	*parC*	*parE*
4	1(1), 5(3)	0.03–0.06	<0.03	B(1), M(3)	–	–	–
8	1(7), 7(1)	0.06–0.125	<0.03	B(1), C(5), E(1), F(1)	–	–	D479E
1	8	0.25	0.125	B	S83Y	–	–
1	1	0.25	0.125	G	D87Y	G83C	D479E
2	2	0.5	0.125	A(2)	D87N	–	S459F, D479E
2	1	0.25	0.125–0.25	L	S83F	–	D479E
1	1	2	0.25	H	S83F	S85R	D479E
4	1	0.5–2	0.25–0.5	K(2), N(1), O(1)	S83F	E89K	D479E
3	1	2–4	0.25–0.5	I	S83V	S85R	D479E
7	1	2–8	0.25–0.5	B(7)	D87H	S85Y	D479E
3	1	2–4	0.25–0.5	B(3)	G75S and S83V	E89K	D479E
1	1	32	2	K	G75S, S83F and D87N	–	D479E
4	1	16–32	2–4	B(1), J(3)	S83F and D87G	–	D479E
2	1	16	4	A(2)	S83F and D87N	S85R	E461D, D479E
2	1	32	4	P(2)	S83F and D87N	S85R	P440S, D479E
3	1	16–32	4–8	B(1), D(2)	S83F and D87Y	S85R	E461K, D479E

[a] 80 μg/ml Phe-Arg-β-naphthylamide.

3.2. Antimicrobial susceptibility testing

Eighteen isolates were resistant to florfenicol. Only one isolate was resistant to ceftiofur.

3.3. Mutations in QRDRs of A. pleuropneumoniae isolates

gyrA, *gyrB*, *parC*, and *parE* mutations were identified by sequencing (Table 2). Overall, seven different substitutions in GyrA (G75S, S83Y, S83F, S83V, D87Y, D87N and D87H), four different substitutions in ParC (G83C, S85R, S85Y and E89K) and five different substitutions in ParE (P440S, S459F, E461D, E461K and D479E) were identified. D479E substitution was the most common (43/48) in ParE, while no mutation was found in GyrB. Twenty-nine isolates with the ER MIC ≧ 2 carried at least one mutation in the *gyrA*, leading to amino acid changes in codon 83 and/or 87, 4 isolates harbored an additional mutation Gly75Ser. The ER MIC values of 12 isolates with double mutations in codon 83 and 87 of *gyrA* (7 isolates carried additional mutations in the codon 85 of the *parC* and codon 440 or 461 of the *parE*) were detected as 16–32 μg/ml.

When the efflux pump inhibitor PAβN was used in the concentration of 80 mg/l, the ER MIC values of the isolates tested decreased (2–16-fold) (Table 2). In the presence of PAβN, *A. pleuropneumoniae* isolates with ER MIC more than 2 μg/ml decreased drastically (4–16-fold) while those of ER MIC less than 2 μg/ml had a slighter decrease (2–4-fold). The ER MICs values of isolates harboring one *gyrA* position substitution (codon 83 or 87) in the presence of PAβN were determined from a concentration range of 0.125–0.5 μg/ml, and isolates with double substitutions in codon 83 and 87 of *gyrA* were determined from a range of 2–8 μg/ml.

4. Discussion

The mechanism of fluoroquinolone action is through binding DNA gyrase and topoisomerase, finally inhibiting bacterial DNA replication (Drlica, 1999). In this study, the mechanisms for enrofloxacin resistance in *A. pleuropneumoniae* strains appear to mainly involve mutations in the GyrA protein (Table 2). Asp479Glu mutation of *parE* found both in susceptible and resistant isolates, suggests that this mutation is possibly not involved in ER resistance. In fact, the exact role of substitutions in ParE has not been well established, and this role needs to be confirmed in future studies. The ER resistance levels of the isolates elevated to >8 μg/ml in the presence of two *gyrA* mutations (Ser83 and Asp87) were probably due to the *gyrA* mutations rather than the *parC* and *parE* mutations. Increasing ER MIC values correlating with stepwise accumulation of mutations in the *gyrA* suggests that DNA gyrase is a primary target of ER in *A. pleuropneumoniae*, as in other Gram negative bacteria. Thus, the *parC* and *parE* mutations might not be directly responsible for ER resistance. ParC and ParE are two subunits of topoisomerase IV homologous with GyrA and GyrB (Hopkins et al., 2005). Topoisomerase IV is the secondary target for quinolones in Gram negative bacteria. Mutations within *parC* or *parE* occurring alone affect the ER susceptibility slightly, but previous studies demonstrated that a mutation in *parC* is the second step leading to high-level FQ resistance in *Salmonella* and *E. coli* (Khodursky et al., 1995; Heisig, 1996). Further studies are necessary to evaluate the contribution of *parC* and *parE* mutations to ER resistance in. *A. pleuropneumoniae*.

Efflux pumps can remove FQs from the cell. To determine how efflux pumps are involved in the ER resistance among the *A. pleuropneumoniae* isolates, susceptibility to ER in the presence of the efflux pump inhibitors at 80 μg/ml PAβN was tested. Differences in the ER MIC values in isolates carrying the same mutation pattern may be influenced by the efflux pumps. When the MICs of ER were determined in the presence of PAβN, the MIC values were uniform, although slight differences could also be observed among isolates carrying mutations, probably associated with efflux pumps not affected by PAβN or difference in the permeability of the outer membrane. These results indicated that efflux pumps contributed to ER resistance for *A. pleuropneumoniae*.

Plasmid mediated quinolone resistance determinants found in isolates from food-producing animals were

reported in China (Yue et al., 2008; Ma et al., 2008; Wu et al., 2008a,b), *qnr* genes in human isolates were also found in Taiwan (Wu et al., 2008a,b). However, these genes were not detected from *A. pleuropneumoniae* isolates in this study. Further investigation is needed to identify if plasmid mediated quinolone resistance determinants exist in *A. pleuropneumoniae*.

In conclusion, this is the first report of fluoroquinolone resistance mechanisms in *A. pleuropneumoniae* isolates. ER resistance of *A. pleuropneumoniae* appears to be linked to multiple target gene mutations at codon positions 75, 83 and 87 of *gyrA*, codon positions 83, 85 and 89 of *parC*, and codon position 440, 459, 461 and 479 of *parE*, as well as being linked to active efflux.

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