Veterinary Microbiology 216 (2018) 159–167

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Characterization of quinolone resistance mechanisms in *Enterobacteriaceae* isolated from companion animals in Europe (ComPath II study)

Anno de Jong[a,b,*], Anaëlle Muggeo[c], Farid El Garch[a,d], Hilde Moyaert[a,e], Christophe de Champs[c], Thomas Guillard[c]

[a] ComPath Study Group, CEESA, B-1150, Brussels, Belgium
[b] Bayer Animal Health GmbH, 51368, Leverkusen, Germany
[c] CHU Reims, Hôpital Robert Debré, Laboratoire de Bactériologie-Virologie-Hygiène, and UFR Médecine, Inserm UMR-S 1250 P3Cell, Université de Reims Champagne-Ardenne, F-51092, Reims, France
[d] Vétoquinol SA, 70204, Lure, France
[e] Zoetis, 1930, Zaventem, Belgium

ARTICLE INFO

Keywords:
Plasmid-mediated quinolone resistance (PMQR)
Quinolone resistance determining region (QRDR)
Fluoroquinolones
qnr
aac(6′)-Ib-cr
oqxAB
qepA
Dogs
Cats

ABSTRACT

ComPath is an ongoing European programme dedicated to monitor antibiotic susceptibility of bacterial pathogens from diseased dogs and cats. The objective was to characterize determinants associated with quinolone resistance among 160 enrofloxacin non-wild type strains (100 *Escherichia coli,* 45 *Proteus mirabilis*, 14 *Klebsiella pneumoniae*, 1 *Enterobacter cloacae*) selected among 843 non-duplicate *Enterobacteriaceae* isolates collected in 12 European countries (2013–2014). These strains with non-wild type MICs of ≥0.25 mg/L, for *P. mirabilis* ≥0.5 mg/L, were screened for PMQR determinants (*qnr, oqxAB*, *qepA* and *aac(6′)-Ib-cr*), and for QRDR mutations in *gyrA*, *gyrB*, *parC* and *parE* genes.

Among them, 20% (32/160) carried at least one PMQR (18/32 *qnrB, qnrS* or *qnrD,* 10/32 *aac(6′)-Ib-cr* and 13/32 *oqxAB*), and 80% (128/160) no PMQR. *qnrB* was detected in 3 *E. coli*, 2 *K. pneumoniae* and 1 *E. cloacae* strains; *qnrS* in 6 *E. coli* and 1 *P. mirabilis* and *aac(6′)-Ib-cr* in 4 *E. coli*, 5 *K. pneumoniae* and 1 *E. cloacae* strains. All *qnrD1* were detected in *P. mirabilis. oqxAB* was detected in 12/14 *K. pneumoniae* and 1 *E. cloacae*. No *qepA* genes were detected. From the 32 PMQR-positive strains, 10 showed enrofloxacin MICs ≤2 mg/L and 22 MICs ≥8 mg/L, the latter carrying 1–4 mutations in QRDR. For the 128 non-PMQR strains, 37 showed enrofloxacin MICs ≤2 mg/L with 0–2 QRDR mutations, and 91 MICs ≥4 mg/L carrying 1–4 QRDR mutations. In conclusion, *qnr* was the major PMQR and *qnrD* only detected in *Proteeae*. Mutations in QRDR play a markedly greater role in mediating fluoroquinolone resistance than PMQR.

1. Introduction

Enterobacteriaceae, primarily *Escherichia coli*, are commonly isolated from clinical samples from dogs and cats, and are considered as major causative agents of their bacterial infections. Several fluoroquinolones, e.g., enrofloxacin and marbofloxacin, have been approved for treatment of canine and feline clinical infections. Resistance to fluoroquinolones is mostly attributed to chromosomal mutations in DNA gyrase and/or topoisomerase IV genes (Quinolone Resistance Determining Region; QRDR) or mutations in the regulatory genes of the efflux pump systems (Piddock, 1999; Hernández et al., 2011; Kang and Woo, 2014). In addition, plasmid-mediated quinolone resistance (PMQR) genes such as the target protection *qnr* genes, the enzymatic modification gene *aac (6′)-Ib-cr* and the efflux pump genes such as *qepA* and *oqxAB,* have also been shown to reduce the susceptibility to fluoroquinolones (Strahilevitz et al., 2009; Poirel et al., 2012). Several studies have been conducted to identify the presence of PMQR in enterobacterial isolates from humans and food animals (Paltansing et al., 2013; Wasyl et al., 2014). However, limited studies describe the prevalence and the role of PMQR determinants and QRDR mutations, such as those found in *gyrA*, *gyrB*, *parC* and *parE*, in mediating resistance in *Enterobacteriaceae* isolates from companion animals.

Under the umbrella of the Executive Animal Health Study Center (Centre Européen d'Etudes pour la Santé Animale; CEESA), research-based veterinary pharmaceutical companies collaborate to organize microbial culture collections and to monitor antimicrobial resistance throughout Europe (de Jong et al., 2013). The CEESA programmes are characterized to be international and applying standardized,

* Corresponding author at: ComPath Study Group, CEESA, B-1150, Brussels, Belgium.
E-mail address: anno.jong-bayer@kpnmail.nl (A. de Jong).

https://doi.org/10.1016/j.vetmic.2018.02.002
Received 19 October 2017; Received in revised form 25 January 2018; Accepted 1 February 2018

quantitative methods and centralized antibiotic susceptibility testing of recovered isolates. CEESA's ComPath is a monitoring programme dedicated to the collection of bacterial pathogens across Europe from diseased dogs and cats not treated recently with antibiotics. The purpose is to determine the antibiotic susceptibility of recovered isolates (Ludwig et al., 2016; Moyaert et al., 2017a).

Objective in this study was to characterize the genetic PMQR and QRDR determinants associated with quinolone resistance among 160 enrofloxacin non-wild type isolates of *Enterobacteriaceae* recovered in the ComPath II monitoring programme.

2. Materials and methods

2.1. Collection of quinolone-resistant strains

Quinolone-resistant isolates were selected from the ComPath II collection. This collection comprises isolates recovered from samples collected in the field by veterinary practitioners in their daily practice during 2013–2014. The design of the ComPath survey including the sampling procedures and the requirements of the collection to be fulfilled have been described previously (de Jong et al., 2013; Guillard et al., 2016). In brief, bacterial isolates were exclusively collected by veterinary practices or clinics from diseased dogs and cats with confirmed clinical diagnoses; subclinical isolates were not accepted. Isolates recovered from animals known to be treated with antibiotics within the four weeks prior to the sampling (or if antibiotic treatment history was unknown) were excluded, as were samples from chronically-diseased animals. In all cases, only one sample per animal was allowed to help prevent the collection of strains that are epidemiologically related. Whenever possible, pets from the same household or pound, cats from the same breeder and dogs from the same kennel were not sampled twice. Veterinarians completed and supplied a sampling form for each isolate tested to confirm compliance with the study protocol. The following 12 countries were selected for sampling: Belgium, Czech Republic, France, Germany, Hungary, Italy, The Netherlands, Poland, Spain, Sweden, Switzerland and the United Kingdom. National laboratories were responsible for recovery and identification of bacterial isolates. However, a complete identification was performed at the central microbiology laboratory, using Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF) technology or biochemical confirmatory tests, if isolates were only identified to genus level by the collecting laboratory.

Among 843 non-duplicate *Enterobacteriaceae* strains collected (554 from urinary tract/prostate infections, 234 from skin/wound/ear infections, 55 from respiratory tract infections), 160 strains (100 *E. coli*, 45 *Proteus mirabilis*, 14 *Klebsiella pneumoniae*, 1 *Enterobacter cloacae*) were selected belonging to non-wild type enrofloxacin phenotype and were screened for PMQR and QRDR characterization. In case of *E. coli*, enrofloxacin non-wild type isolates were selected with MICs to enrofloxacin exceeding epidemiological cut-off (ECOFF) values as defined by EUCAST ($\geq$0.25 mg/L). As for the other *Enterobacteriaceae* species included, no interpretive criteria are set by EUCAST (www.eucast.org), the same selection criterion as for *E. coli* ($\geq$0.25 mg/L) was applied for these species. For *P. mirabilis* a cut-off of $\geq$0.5 mg/L was applied based on the deviating wild type population of this specie. Isolates were identified to genus and species level using conventional methods such as colony morphology, Gram staining, production of indole and biochemical tests (API systems; BioMérieux, Marcy l'Etoile, France). Identifications of all selected isolates were verified using MALDI-TOF MS Microflex LT (Bruker Daltonics, Bremen, Germany). MIC values were determined by agar dilution according to Clinical and Laboratory Standards Institute standards (CLSI, 2013) as reported elsewhere (Moyaert et al., 2017b,c; de Jong et al., 2017).

2.2. Molecular detection of PMQR and QRDR

Strains were screened for *qnr*, *qepA*, and *oqxAB* genes by real-time PCR; *aac(6′)-Ib-cr* was detected by pyrosequencing as described elsewhere (Guillard et al., 2010, 2011, 2015). High-resolution melting analysis was used for characterization of *qnr* alleles (Guillard et al., 2012a,b). For *qnrD*, plasmids were fully sequenced using the same strategy as previously described for pDIJ09-518a (Guillard et al., 2012b). Hot spots of mutation in the *gyrA*, *gyrB*, *parC* and *parE* genes were characterized using a pyrosequencing-based approach, as described elsewhere (Guillard et al., 2016). In all runs, previously identified PMQR-positive strains were included as positive controls.

3. Results

3.1. Total collection

Among the 160 enrofloxacin non-wild type *Enterobacteriaceae* isolates, 32 isolates (20.0%) harboured PMQR determinants (Table 1) and in 128 isolates (80.0%) no PMQR was found (Table 2). Interestingly, depending on the species, most of the PMQR and non-PMQR isolates carried QRDR mutations (n = 93/100; 93.0%, n = 13/14; 92.9% and n = 38/45; 84.4% for *E. coli*, *K. pneumoniae* and *P. mirabilis*, respectively), and the *E. cloacae* isolate carried both PMQR and a QRDR mutation. As shown in Table 1, *qnr* gene was the major PMQR determinant found in this study. Among the 32 PMQR strains, 21 strains were recovered from urinary tract infections, 4 from respiratory tract infections, 5 from ear infections and 2 isolates from wound infections. From each participating country except Sweden, at least one isolate harboured PMQR. A relationship between the number of PMQR determinants and enrofloxacin MIC values was not apparent. The same applied for the MIC values of marbofloxacin, another fluoroquinolone tested (data not shown). In contrast, for QRDR, especially *E. coli*, a clear relationship between number of mutations and enrofloxacin MIC values was observed.

3.2. PMQR genes

Fourty-two PMQR determinants were detected with a large majority of *qnr* genes (45.2%) before *oqxAB* (31.0%) and *aac(6′)-Ib-cr* (23.8%) (Fig. 1A). Among the 32 PMQR-producing strains, 19 *qnr* genes (59.4%), 10 *aac(6′)-Ib-cr* (31.3%) and 13 (40.6%) *oqxAB* genes were detected. Seven *qnrS* genes (21.9%) were detected with 6 *qnrS1* (5 *E. coli* and 1 *K. pneumoniae)* and 1 *qnrS2 (P. mirabilis)*. Six *qnrB* genes (18.8%) were detected with allele characterization as follow: 3 *qnrB1* (*K. pneumoniae; E. cloacae*), 1 *qnrB4* (*E. coli*), and 2 *qnrB10* (*E. coli*). Six *qnrD* genes (18.8%) were detected and only in *P. mirabilis* strains with sequenced allele 100% identical to *qnrD1* allele (Cavaco et al., 2009). All *qnrD* genes were harboured onto small non-transmissible plasmid similar to pDIJ09-518a (Guillard et al., 2012b). Neither *qnrA* or *qnrC* nor *qepA* were detected. Eleven *aac(6′)-Ib-cr* genes (34.4%) were detected in 4 *E. coli,* 6 *K. pneumoniae* and 1 *E. cloacae*. All 12 *K. pneumoniae* and *E. cloacae* strains carried *oqxAB* genes.

3.3. PMQR in bacteria

Of the 100 *E. coli* strains, 5 harboured *qnrS1* (5%), 3 harboured *qnrB* genes (3%; *qnrB4* and *qnrB10*) and 4 strains, all without *qnr*, harboured *aac(6′)-Ib-cr* (4%) (Fig. 1B). Among the 45 *Proteeae* strains, *qnr* genes were found in 7 *P. mirabilis* strains (6 with *qnrD1* and 1 with *qnrS2),* and the plasmids were similar to those previously described (i.e. pDIJ09-518a). Five plasmids (pCEESA17.8, pCEESA17.35, pCEESA17.36, pCEESA17.108 and pCEESA17.149) were 2683 bp long with 3–4 single nucleotide polymorphisms (SNP). Among these SNPs only one led to an amino acid substitution in ORF3 (T121I). The last *qnrD* plasmid (pCEESA17.70) was a 2536 bp-plasmid showing many differences compared to pDIJ09-518a. Indeed, it carried 281 nucleotides differences and 2 deletions (24 nucleotides and 135 nucleotides) between ORF3 and ORF4. The SNPs led to 8 amino acid substitutions in ORF3, 1