Extended-Spectrum-Beta-Lactamases, AmpC Beta-Lactamases and Plasmid Mediated Quinolone Resistance in Klebsiella spp. from Companion Animals in Italy

Donati, Valentina; Feltrin, Fabiola; Hendriksen, Rene S.; Svendsen, Christina Aaby; Cordaro, Gessica; Garcia-Fernandez, Aurora; Lorenzetti, Serena; Lorenzetti, Raniero; Battisti, Antonio; Franco, Alessia

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Introduction

*Klebsiella* are bacterial pathogens that can cause a variety of severe infections in humans, mainly due to *K. pneumoniae* (KP) [1], [2] and to a lesser degree to *K. oxytoca* (KO) [3], [4]. KP is also a well-known causal agent of mastitis in cattle and bacteremia in calves, cervicitis and metritis in mares, pneumonia and septicaemia in foals, pneumonia, urinary tract infection (UTI) and septicemia in dogs [5], [6], [7].

Increasing antimicrobial resistance, especially towards aminoglycodies, (fluoro)quinolones, third and fourth generation cephalosporins, cephamycins, and carbapenems have been reported in the last decade [8], [9], [10], and poses serious therapeutic problems when treating *Klebsiella* infections in humans. In veterinary medicine, scarce information is reported on the occurrence of extended spectrum beta-lactamases (ESBLs), AmpC beta-lactamases and plasmid mediated quinolone resistance (PMQR) in *Klebsiella* isolates from companion animals [11], [12]. The aim of the study was to provide molecular characterization of extended-spectrum cephalosporin (ESC) resistance and PMQR in *Klebsiella* isolates from clinical cases or lesions in necropsied animals of canine and feline origin in Italy. A further aim was to determine phenotype and genotype of co-resistances, and to provide plasmid identification and genetic relatedness by Multilocus Sequence Typing (MLST) and Pulsed Field Gel Electrophoresis (PFGE) among the isolates, to evaluate potential clustering of ESC, PMQR, and other resistance genes among clones.

Materials and Methods

Origin of ESC-resistant Klebsiella

Between 2006 and 2012, the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT) investigated samples from 1555 dogs and 429 cats of clinical cases and necropsy specimens with suspicious bacterial infections, submitted by veterinarians practising mainly in central Italy, and some practising in northern Italy. Presumptive positive *Klebsiella* isolates were identified using the API 20E identification system (bioMérieux, Graponne, France). For species-level identification of isolates
with phenotypic inconclusive results 16S rDNA sequencing technique was employed, by means of the MicroSeq Full Gene system (Applied Biosystems, USA) as described previously [13].

Genotypic characterization

Multilocus Sequence Typing on KP isolates was performed as previously described [14], and interpreted according to the KP MLST database (www.pasteur.fr/mlst).

In addition, all isolates were genotyped by PFGE using XbaI according to the previously published protocol [15].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed as minimum inhibitory concentrations (MIC) by micro-broth dilution in 96-well microtitre plates [Trek Diagnostic Systems, Westlake, OH, USA]. The following antimicrobials were tested: ampicillin, cefotaxime, cefazidime, ciprofloxacin, chloramphenicol, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfonamides, tetracycline, and trimethoprim. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-offs (www.eucast.org) and to Clinical Laboratory Standard Institute [16] or EUCAST clinical breakpoints for those drugs for which epidemiological cut-offs have not been made available (kanamycin, chloramphenicol, sulfamethoxazole, trimethoprim). For streptomycin, a cut-off of 16 mg/L was used, according to EUCAST MIC distributions.

Confimatory test for the detection of ESBLs were performed on isolates resistant to cefotaxime or cefazidime according to Clinical Laboratory Standard Institute (CLSI) recommendations [16].

Detection of genes encoding beta-lactamase and PMQR

For the confirmed ESBL-producing isolates, the encoding genes belonging to the beta-lactamase and PMQR families were further analyzed for the presence of blaCTX-M [17], blaSHV [18], blbTEM [19], blbOXA [20], blaAmnC families [21], as well as for genes of the qnr family, qep-A, and aac(6’)-Ib-cr encoding for PMQR [22], [23], [24], [25], [26]. The isolates were further screened by PCR for genes encoding carbapenemases [27]. Amplicons were sequenced by BigDye Terminator chemistry (Applied Biosystems, Foster City, CA, USA) and migrated with an automated sequencer (ABI Prism 310; Applied Biosystems). Sequence data analysis was performed using CLC DNA workbench software version 5.7.1 (CLC Bio, Aarhus, Denmark) and evaluated against the GenBank nucleotide databases.

Detection of plasmid replicons

Identification of plasmids was performed by PCR-based replicon typing as previously described [28], [29], [30], and using the PBRT kit (Diatheva, Fano, Italy).

Plasmid analysis

Plasmid DNA preparations were performed using the NucleoSpin Plasmid/Plasmid (NoLid) kit (Macherey-Nagel, Düren, Deutschland) and used to transform MAX Efficiency DH5α Competent Cells (Invitrogen, Life Technologies, U.S.A). In order to identify the plasmids carrying the ESBLs and AmpC genes, the selection of the transformants was performed on LB agar plates containing 100 mg/ml ampicillin.

Additionally, the isolates were tested according to the manufacturer’s instructions using an array hybridization kit for DNA-based detection of the most common resistance genes, and for the integrase gene (intI1) of class 1 integrons of Gram negative bacteria (Alere Technologies GmbH, Jena, Germany) and the results interpreted by the ArrayMate, Alere.

Results

Isolation rates

The samples (n = 1984; dogs and cats) yielded a total 70 (3.53%, 95% CI: 2.72%–4.34%) KP and 23 (1.16%), 95% CI: 0.69%–1.63%) KO among the isolates, respectively. Of these, 15 (21.4%) KP and four (17.4%) KO revealed resistance to ESC and were investigated in this study.

Genetic relatedness

The 15 KP isolates investigated by MLST were assigned to four different Sequence Types (ST): ST11 (n = 1), ST340 (n = 2), ST101 (n = 8), and ST15 (n= 4) (Figure 1). ST11 and its single-locus (mutB) variant (SLV) ST340 (3/15, 20%), both belonging to CC11, were detected in 2012. The separation of the isolates based on MLST corresponded well with PFGE results grouping the same isolates (Figure 1). A total of 11 different PFGE patterns were observed including two clusters of two and four indistinguishable isolates, respectively (Figure 1). The cluster of the two isolates both belonged to ST340 and was related (80% similarity) to a single isolate exhibiting a unique PFGE pattern and belonging to ST11. The other cluster of four indistinguishable isolates was highly related (from 99% to 90% similarity) to additional four isolates within the same PFGE group, all belonging to ST101 (Figure 1). No clustering was observed related to time, animal origin, nor infection, but some to the presence of resistance genes (Table 1).

No MLST was assigned to the four KO isolates. However, the four isolates revealed three different PFGE patterns of which one was a cluster of two identical isolates (Figure 2). The three patterns seemed not to be related, indicating a similarity of 45% and 55% to the pattern of the two clustering isolates. Interestingly, the two isolates of the same PFGE pattern were both from dogs and isolated within the same year, but it could be the result of a random effect (Figure 2).

Antimicrobial susceptibility testing

All isolates showed microbiological resistance to third-generation cephalosporins, and also clinical resistance, either when the MIC results were interpreted according to clinical breakpoints set by CLSI [16] or by EUCAST (e.g. MIC cefotaxime ≥ 4mg/L), except for 9KP (MIC 1mg/L). The phenotype for PMQR was evident (ciprofloxacin MIC 0.25mg/L, nalidixic acid 8mg/L) in KO isolates only, because in all KP isolates it was masked by concurrent genetic background conferring Mics of 8 and 128mg/L, respectively.

Moreover, all isolates showed multidrug-resistance towards other classes of antimicrobials, such as aminoglycosides, sulfonamides, tetracyclines, dihydrofolate reductase inhibitors and amphenicols, mediated by strA/B, adaA2, adaB, ant (2’)-In, aac(6’)-Ib, sul, tet, dfr and cat genes in various combinations, as reported in Table 1.

Genes encoding ESBL-, AmpC-, and PMQR

All Klebsiella isolates investigated showed the presence of at least one ESBL or AmpC gene encoding ESC resistance. Additionally, 16 out of 19 isolates harbored a PMQR gene (qnr family or aac(6’)-Ib-cr, single or in combination). The most frequent ESC gene harbored by KP isolates was blaCTX-M-15 (n = 11, 58%), detected in all eight ST101, in one ST13 and in both two ST340 isolates, respectively. All four ST15 KP isolates carried the blaSHV-28 gene, single or in combination with the ESC resistance genes blaCTX-M-15.


**Figure 1. Dendrogram showing the genotypic relatedness of ESC-resistant *Klebsiella pneumoniae* (KP) isolates from dogs and cats based on XbaI-PFGE fingerprints, and comparison with Multilocus Sequence Typing classification.**

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**Table 1.** Sequence Types, plasmid incompatibility groups, and antimicrobial resistance phenotypes and genotypes in ESC-resistant *Klebsiella pneumoniae* (KP) and *Klebsiella oxytoca* (KO).

<table>
<thead>
<tr>
<th>Sequence Type</th>
<th>Key</th>
<th>Antimicrobial Resistance profile</th>
<th>Plasmid</th>
<th>ESBL and AmpC genes</th>
<th>PMQR genes</th>
<th>Other resistance and Integron genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>2KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncI1; IncR</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>aadA2; catA1; dfrA12; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>15</td>
<td>9KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncR; IncFIIk</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>strA/B; catA1; dfrA12; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>15</td>
<td>7KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncR</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>aadA2; strB; catA1; dfrA12; sul1; tet(A)</td>
</tr>
<tr>
<td>15</td>
<td>13KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncN; IncFIIk; IncFIA; IncR</td>
<td>blaCTX-M-1; blaSHV-28;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>aadA2; dfrA1; dfrA14; dfrA16; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>10KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>aadA2; strB; catA1; dfrA12; sul1; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>3KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>strA/B; dfrA14; sul1; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>5KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>aadA2; strB; catA1; dfrA12; sul1; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>6KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncH2; IncR; IncFIIk</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>strA/B; dfrA14; sul1; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>4KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>strA/B; dfrA14; sul1; tet(A)</td>
</tr>
<tr>
<td>101</td>
<td>1KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI</td>
<td>IncH2; IncR; IncFIIk</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; intI1</td>
<td>strA/B; dfrA14; sul1; tet(A)</td>
</tr>
<tr>
<td>340</td>
<td>17KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncH2; IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; qnrS1</td>
<td>aadA2; dfrA1; dfrA14; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>340</td>
<td>18KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncR; IncFIIk; IncFII</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>aac(6’)-Ib-cr; qnrS1</td>
<td>aadA2; dfrA1; dfrA14; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>11</td>
<td>16KP</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncN; IncFIIk</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>qnrS1</td>
<td>aadA2; dfrA1; dfrA14; sul1; tet(A)</td>
</tr>
<tr>
<td>NA</td>
<td>3A KO</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncH2; IncLM</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>qnrS1</td>
<td>strA/B; dfrA14; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>NA</td>
<td>4A KO</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncH2; IncLM</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>qnrS1</td>
<td>strA/B; dfrA14; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>NA</td>
<td>1A KO</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncH2; IncP</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>qnrS1</td>
<td>aadA4; dfrA1; dfrA14; sul1; tet(A); intI1</td>
</tr>
<tr>
<td>NA</td>
<td>6A KO</td>
<td>AMP,CTX,CFT,NAL,CIP,STR,KAN,CLO,SULFA,TRI,TET</td>
<td>IncH2</td>
<td>blaCTX-M-15; blaSHV-1; blaTEM-1; blaOXA-1;</td>
<td>qnrS1</td>
<td>aadA2; dfrA1; dfrA14; sul1; tet(A); intI1</td>
</tr>
</tbody>
</table>

Legend:
NA: Not Applicable; AMP = Ampicillin; CFT = Ceftazidime; CIP = Ciprofloxacin; CLO = Chloramphenicol; CTX = Cefotaxime; GEN = Gentamicin; KAN = Kanamycin; NAL = Nalidixic Acid; STR = Streptomycin; SULFA = Sulfamethoxazole; TET = Tetracycline; TRI = Trimethoprim.

Note: When underscored, plasmids and their content of beta-lactamase and PMQR genes where detected in transformant strains.
time in clinical cases of pets from Italy the clone KP ST11 and its
SLV ST340, harboring ESC and qnrST-PMQR resistance. Among
these CC11 isolates, the ST11 harbored IncN plasmid, which has
been frequently involved in the transmission of the blaCTX-M-1
gene, a feature suggesting an animal reservoir for this ESBL, since
this Inc plasmid types have been demonstrated to be highly prevalent
in zoonotic enterobacterial pathogens [29]. The same animal origin
reservoir is proposed for the IncH1 plasmids harboring the
blaKPC-2 gene found in E. coli avian commensal strains [34].

It is noteworthy that ST11 and ST340 carried transferable
ESBL resistance but not resistance to carbapenems. ST(CC)11
and ST15 and ST101 are among human epidemic clones,
carrying both ESBLs and carbapenemases, which have been
increasingly detected worldwide, in Europe and in Italy in the last
years [36], [37], [38], [39], [40].

These infections are worrisome, since the antimicrobial
treatment options for these multidrug-resistant strains are very
limited. In Italy, during the last years the rapid emergence of the
carbapenemase KPC-producing KP, belonging to the ST101,
CC11, and predominantly to a single Sequence Type ST258, has
become a serious problem in health-care settings [41], [42], [43].

As for CTX-M and SHV-12 ESBLs in Italy, a high occurrence
in KP isolated from humans has been demonstrated, being the
ST15, ST37, ST147 and ST273 the prevalent clones [44], [45],
[46], [47].

In two KO isolates, the ESBL-encoding blaSHV-12 gene co-
existed with the AmpC gene blaSHV-1 in accordance with
phenotype of resistance to cefotaxime and cefoxitin observed in
the ESBL phenotypic confirmatory test. These two isolates also
carried the PMQR gene qnrB4. In these two KO transformants the
IncL/M plasmid harbored both the blaSHV-12 and the blaDHA-1
genes but not the qnrB4 gene. To our knowledge, this feature has
never been described before.

The other KO presented the blaCTX-M-9 gene, located in an
IncHI2 plasmid as described worldwide but associated to a qnrA1
gene, a feature previously described in Spain in E. coli and KP of
human origin [48], [49] and in KO in clinical specimens from
Japan [50].

Similarly to what has been observed in other human and canine
KP isolates [34], [12], the association of blaCTX-M genes with the
the aac(6’)-Ib-cr encoding an aminoglycoside acetyl transferase
determining PMQR, was demonstrated in all ST101 and ST340
isolates, but only in one out of four ST15, but these PMQR genes
were not located in the same plasmid in our strains. Conversely,
PMQR encoded by different qnr genes of the qnrC or qnrB groups
were observed in all the KO isolates studied (Table 1). In the case
of the qnrC1 gene, the two KO isolates also harbored the ESBL
blaCTX-M-9 gene, a feature reported previously in association with
blaCTX-M-1 and IncHI2 plasmids in KO of human origin [51].

Multidrug-resistance in the ESC resistant and PMQR isolates
studied is of further concern from a therapeutic perspective, for a
possible impact on clinical outcome of affected animals. In many
isolates, the demonstration of the integrate intI1, accounts for
the presence of resistance gene cassettes with aac, adaA, cat, dfr genes,
associated with Class I integrons, similarly to what has been
described in KP of human origin [52], [53]. As for streptomycin
resistance, MIC>16 mg/L correlated in 100% isolates with the
presence of strA/B genes.

Fortunately, the absence of carbapenemases offers so far a
better scenario for antimicrobial therapy in companion animals,
although a possible circulation, within a short time, of these
carbapenemase-producing epidemic strains, is of concern also in
veterinary medicine.

In conclusion, monitoring and characterization of multidrug-
resistant Klebsiella in companion animals by means of phenotypic
and molecular methods proved to be useful for providing a picture
of mechanisms of resistance that may further spread clonally or by
horizontal gene transfer, at regional or even at international level.
Sharing this kind of information appears essential for building
awareness in companion animal therapy, also in view of preventing
and controlling the spread of multidrug-resistant strains in veterinary hospital settings. Indeed, the bi-directional
exchange between owners and pets of Klebsiella carrying resistance to critically important antimicrobials for human health, raise some
concerns also for the possibility of a spill back to humans, especially at household level.

The emergence of the PMQR and, above all, the emergence of
current transferable cephamycin, oxymino-cephalosporin, and
beta-lactamase inhibitor resistance in multidrug-resistant
Klebsiella isolates in pets, a recent issue even in human therapy [54], may
pose in the next future further and serious therapeutic challenges also in bacterial infections of companion animals.

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Author Contributions
Conceived and designed the experiments: AF AB RSH FF AGF.
Performed the experiments: VD SL FF GC RL CAS. Analyzed the data:
VD FF AF AB RL RSH CAS AGF. Contributed reagents/materials/
analysis tools: AB AF RSH. Wrote the paper: AF AB RSH AGF.
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