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Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in dogs and cats with urinary tract infection: 16 year retrospective study

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Objectives: To evaluate temporal trends in antimicrobial resistance, over 16 years, in bacteria isolated from dogs and cats with urinary tract infection (UTI) and the clonal lineages of bacteria harbouring critical antimicrobial resistance mechanisms.

Methods: Antimicrobial susceptibility testing was conducted for 948 bacteria isolated from dogs and cats with UTI (1999–2014). Resistance mechanisms were detected by PCR, namely ESBL/AmpC in third-generation cephalosporin (3GC)-resistant *Escherichia coli* and *Proteus mirabilis*, *mecA* in methicillin-resistant staphylococci, and *aac(6′)-Ieaph(2″)-Ia* and *aph(2″)-1d* in high-level gentamicin-resistant (HLGR) enterococci. Resistant bacteria were typed by MLST, and temporal trends in *E. coli* and Enterobacteriaceae antimicrobial resistance were determined by logistic regression.

Results: Enterobacteriaceae had a significant temporal increase in resistance to amoxicillin/clavulanate, 3GCs, trimethoprim/sulfamethoxazole, fluoroquinolones, gentamicin and tetracycline ($P < 0.001$). An increase in MDR was also detected ($P < 0.0001$). 3GC resistance was mainly caused by the presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ in *E. coli* and the presence of $bla_{\text{CMY-2}}$ in *P. mirabilis*. Two major 3GC-resistant *E. coli* clonal lineages were detected: O25b:H4-B2-ST131 and ST648. The *mecA* gene was detected in 9.2% ($n = 11/119$) of *Staphylococcus* spp., including MRSA clonal complex (CC) 5 ($n = 2$) and methicillin-resistant *Staphylococcus epidermidis* CC5 ($n = 4$). A temporal increase in MDR methicillin-resistant *Staphylococcus pseudintermedius* was detected ($P = 0.0069$). Some ampicillin-resistant and/or HLGR *Enterococcus* spp. were found to belong to hospital-adapted CCs, namely *Enterococcus faecalis* ST6-CC6 ($n = 1$) and *Enterococcus faecium* CC17 ($n = 8$).

Conclusions: The temporal increase in antimicrobial resistance and in MDR bacteria causing UTI in dogs and cats creates important therapeutic limitations in veterinary medicine. Furthermore, the detection of MDR high-risk clonal lineages raises public health concerns since companion animals with UTI may contribute to the spread of such bacteria.

Introduction

Urinary tract infections (UTIs) are frequently diagnosed in veterinary medicine[1] and may require antimicrobial treatment.[2] Since antimicrobial resistance is known to change geographically and over time,[3] updated and long-term studies are critical to investigate the spread of antimicrobial resistance. *Escherichia coli* and *Proteus mirabilis* are the most frequently isolated Gram-negative bacteria from dogs and cats with UTI, while *Staphylococcus* spp. and *Enterococcus* spp. are the most common Gram-positive bacteria.[1,4] These bacteria, isolated from dogs and cats, may harbour clinically and epidemiologically important resistance mechanisms of human and veterinary relevance such as ESBL,[5,6] cephalosporinases (AmpC),[7] PBP2a[8] and high-level gentamicin resistance (HLGR) bifunctional enzyme.[9] Moreover, the detection of MDR bacteria in dogs and cats is being increasingly reported,[7,8] posing a difficult veterinary therapeutic challenge and often requiring the use of antimicrobials critically important to humans.[10] With the growing contact between companion animals and humans, the risk of animal-to-human transfer of such bacteria is of concern.[11] Additionally, several studies have shown that dogs and cats may share uropathogenic bacteria with the remaining household members.[12] Therefore, the identification of the clonal lineages of

bacteria isolated from dogs and cats with UTI, especially those harbouring important resistance mechanisms, is crucial to evaluate the extent to which dogs and cats with UTI may act as reservoirs for resistant bacteria.

The goal of this study was to determine the temporal trends of antimicrobial resistance of bacteria isolated from dogs and cats with UTI over 16 years and to characterize their major antimicrobial resistance mechanisms, namely ESBL and AmpC in *E. coli* and *P. mirabilis*, methicillin resistance in *Staphylococcus* spp., and ampicillin and HLGR in *Enterococcus* spp. Furthermore, this study aimed to determine the clonal lineages of *E. coli*, *Staphylococcus* spp. and *Enterococcus* spp. harbouring such resistance genes and hence evaluate their potential public health relevance.

Materials and methods

Bacterial isolates

A total of 948 consecutive positive bacterial isolates from dogs and cats with UTI ($n = 869$), collected from 1999 to 2014 at the Laboratory of Antimicrobial Resistance from the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine/University of Lisbon and from several private practices in the Lisbon region were included in this study. Samples were collected by cystocentesis, catheterization or free catch as part of the routine care of dogs and cats with UTI. UTI was diagnosed based on clinical and urine cytological findings together with the detection of significant bacteriuria by quantitative urine culture.

Bacteriological methods

Quantitative urine culture was performed. Briefly, 10 μL of urine was inoculated onto 5% sheep blood (bioMérieux, Marcy-l'Étoile, France) and MacConkey (Biokar Diagnostics, Allonne, France) agar plates. After incubation at 37°C for 24–48 h under atmospheric conditions, colonies were quantified and scored as having significant bacteriuria according to the urine sample collection method as defined elsewhere.[13] Samples were included in the study regardless of the type of UTI.

Species identification was conducted by phenotypic tests (API, bioMérieux and BD™ BBL™ Crystal Gram Positive ID Kit, Becton Dickinson, MD, USA). At the time of collection, isolated bacteria were stored in 20% glycerol (Sigma–Aldrich, St Louis, MO, USA) brain heart infusion broth (Biokar Diagnostics) at −80°C for future studies.

For the present study, stored isolates were recovered by streaking them onto 5% sheep blood agar. Whenever recovery was not possible, existing antimicrobial susceptibility results were used. *E. coli*,[14] *Klebsiella* spp.,[15,16] *Proteus* spp.,[17] *Enterococcus* spp.[18] and *Staphylococcus* spp.[8] were confirmed by PCR and/or sequencing of 16S rRNA.

Susceptibility testing

Susceptibility testing was performed by the disc diffusion method according to CLSI guidelines, and *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used for quality control.[19] The following antimicrobials were tested (Oxoid, Hampshire, UK): amoxicillin/clavulanate 30 μg, cefotaxime 30 μg, cefovecin 30 μg, cefoxitin 30 μg, ceftazidime 30 μg, ciprofloxacin 5 μg, enrofloxacin 5 μg, gentamicin 10 μg, high-level gentamicin 120 μg, oxacillin 1 μg, penicillin 10 U, tetracycline 30 μg and trimethoprim/sulfamethoxazole 25 μg. Veterinary CLSI breakpoints[20] were used for amoxicillin/clavulanate, cefoxitin, enrofloxacin, gentamicin, high-level gentamicin, oxacillin, penicillin, tetracycline and trimethoprim/sulfamethoxazole; human CLSI breakpoints[21] were used for cefotaxime, ceftazidime and ciprofloxacin. Finally, cefovecin results were interpreted according to the manufacturer's breakpoints. Initial ESBL screening was conducted by a double-disc synergy test.[19] Cefoxitin or oxacillin were used to predict methicillin resistance in *Staphylococcus* spp. according to CLSI guidelines.[20,21]

DNA extraction, sample purification and sequencing

DNA extraction was conducted using a boiling method.[5] For PCR amplicon sequencing, DNA purification was conducted using a NZYTech Gel Pure Kit (NZYTech—Genes and Enzymes, Lisbon, Portugal) and sequencing was performed by Stabvida (Caparica, Portugal). Sequences were analysed using Ugene Unipro software (Unipro, Novosibirsk, Russia) and the nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/).

Antimicrobial resistance genes

Third-generation cephalosporin (3GC)-resistant *E. coli* and *P. mirabilis* were screened for the presence of ESBL $bla_{\text{CTX-M-type}}$ and AmpC $bla_{\text{CIT-type}}$, $bla_{\text{DHA-type}}$, $bla_{\text{MOX-type}}$, $bla_{\text{ACT-type}}$, $bla_{\text{FOX-type}}$ and $bla_{\text{MIR-type}}$ by PCR and sequencing.[22–24] 3GC-resistant Enterobacteriaceae without $bla_{\text{CTX-M-type}}$ or AmpC genes were further tested for the presence of $bla_{\text{TEM-type}}$ and $bla_{\text{SHV-type}}$ ESBL genes.[5] *Staphylococcus* spp. were screened for the presence of the *mecA* gene[8] and HLGR *Enterococcus* spp. for the presence of *aac(6′)-Ieaph(2″)-Ia* and *aph(2″)-1d*[25] genes by PCR. Negative and previously sequenced positive controls were included in all PCR reactions.

Clonal lineages of resistant isolates

ESBL- or AmpC-producing *E. coli* were tested for the clonal lineage O25b:H4-B2-ST131 by PCR.[14,26] 3GC-resistant *E. coli* isolates not belonging to the O25b:H4-B2-ST131 clonal lineage were typed by MLST.[27] Methicillin-resistant *Staphylococcus* spp. were previously characterized by MLST, SCC*mec* and spa typing elsewhere.[8] Ampicillin-resistant and/or HLGR *Enterococcus faecium* and *Enterococcus faecalis* were also typed by MLST.[28,29] eBURST v. 3 software (http://eburst.mlst.net/) was used to estimate the relationships between the isolate STs from this study and all MLST profiles known to date.

Statistical analysis

The SAS statistical software package for Windows v. 9.3 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Antimicrobial resistance frequencies were only calculated if at least 10 isolates were tested for a specific organism/antimicrobial combination and results were presented with the 95% CI. For statistical purposes, intermediate isolates were considered susceptible. An isolate was considered resistant to 3GCs when it was found to be resistant to at least one of the three 3GCs tested (cefotaxime, ceftazidime and cefovecin). Ciprofloxacin and enrofloxacin were used as markers of fluoroquinolone resistance. An isolate was considered MDR when it was found to be fully resistant to three or more antimicrobial categories. The antimicrobial categories were adapted from those proposed by other authors[30] and varied according to the bacterial species considered (Table S1, available as Supplementary data at *JAC* Online).

When at least 10 isolates were tested per year, temporal trends of antimicrobial resistance were determined using an SAS LOGISTIC regression model with the year as a continuous variable and an α value of 0.05. Thus, temporal trends in antimicrobial resistance were determined for *E. coli* and for Enterobacteriaceae (including *E. coli*, *Proteus* spp., *Klebsiella* spp. and *Enterobacter* spp. as a group). When determining Enterobacteriaceae temporal trends, intrinsic resistance data was excluded from analysis. Furthermore, *P. mirabilis* and *Staphylococcus* spp. antimicrobial resistance were compared between two time periods: 1999–2006 and 2007–14. The Fisher's exact test was used for comparisons between groups with an α value of 0.05.

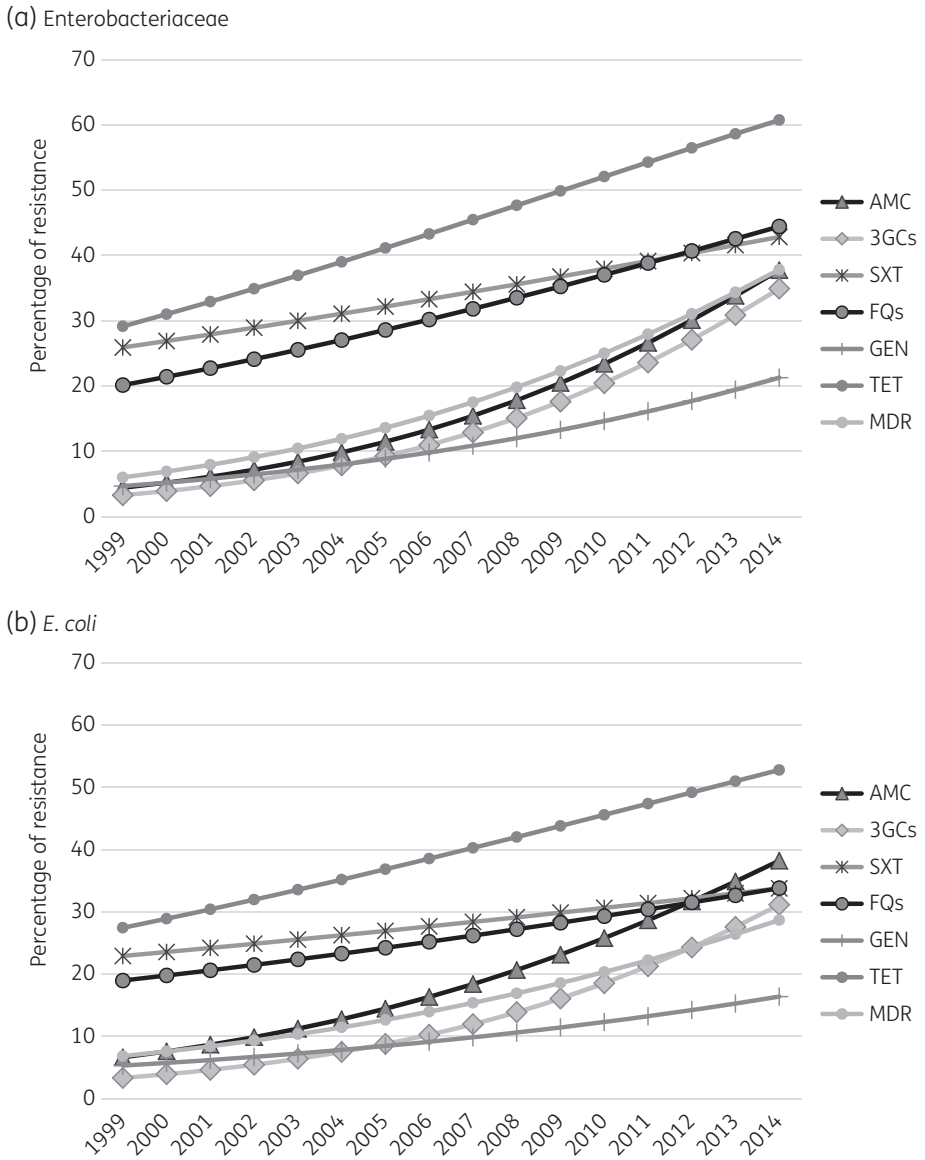

Figure 1. Enterobacteriaceae and *E. coli* resistance trends over the 16 years of the study. Depiction of the temporal trends in resistance obtained by logistic regression over 16 years. (a) Enterobacteriaceae. There was a significant increase ($P < 0.05$) in antimicrobial resistance to all antimicrobials. (b) *E. coli*. There was a significant increase ($P < 0.05$) in antimicrobial resistance to all antimicrobials, except trimethoprim/sulfamethoxazole. AMC, amoxicillin/clavulanate; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline.

Results

From 1999 to 2014, 948 bacteria were isolated from 649 dogs and 220 cats with UTI. The majority of UTIs (91.1%, CI 89.2%–93.0%, $n = 792/869$) were caused by single organisms. Coinfections were most commonly caused by the combinations of *E. coli*/*Enterococcus* spp. (14.3%, CI 6.5%–22.1%, $n = 11/77$), *E. coli*/*P. mirabilis* (11.7%, CI 4.5%–18.9%, $n = 9/77$) and *E. coli*/*Streptococcus* spp. (10.4%, CI 3.6%–17.2%, $n = 8/77$).

Although *E. coli* (43.5%) was the most frequently isolated bacterium, *Proteus* spp. (16.4%), *Staphylococcus* spp. (13.2%) and *Enterococcus* spp. (7.0%) were also common (Table S2). The frequency of infection by *Proteus* spp. was significantly higher ($P < 0.0001$) in dogs and *Enterococcus* spp. in cats, respectively (Table S2). Overall, *Staphylococcus* spp. had similar frequencies in cats and dogs. However, *Staphylococcus pseudintermedius* was significantly more common in dogs ($P < 0.0001$), while cats were infected by a higher diversity of staphylococcal species, with *S. pseudintermedius*, *Staphylococcus felis* and *Staphylococcus epidermidis* being the most frequent (Table S2).

Enterobacteriaceae accounted for 66.1% (CI 63.1%–69.2%) of all isolated bacteria and showed a significant temporal increase in resistance to all tested antimicrobials (Table S3 and Figure 1). In 2012–14, resistance of Enterobacteriaceae to all the antimicrobials tested, except gentamicin, was >30% (Figure 1). Considering *E. coli* and *Proteus* spp. alone, no significant change over time was detected in trimethoprim/sulfamethoxazole resistance (Tables S3 and S4).

Table 1. CTX-M-producing *E. coli* clonal lineages

Isolate	Year	β-Lactamase	Clonal lineage	CC	Animal	MDR	AMC	3GCs	SXT	FQs	GEN	TET
FMV5825/04	2004	CTX-M-15	ST131:H4-B2-O25b	CC131	dog	yes	R	R	R	R	R	R
FMV521/07	2007	CTX-M-32	ST224	—	cat	likely	I	R	S	R	S	R
FMV1630/07	2007	CTX-M-15	unassigned ST[b]	CC23	dog	yes	I	R	R	R	S	R
FMV7261/07	2007	CTX-M-32	ST609	CC46	dog	yes	I	R	R	R	S	R
FMV635/08	2008	CTX-M-32	ST23	CC23	cat	yes	R	R	R	R	S	S
FMV2777/08	2008	CTX-M-15	ST131:H4-B2-O25b	CC131	cat	yes	R	R	S	R	R	R
FMV5827/08	2008	CTX-M-15	ST23	CC23	dog	yes	R	R	R	R	S	S
FMV1952/10[a]	2010	CTX-M-9	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV4479/13[a]	2013	CTX-M-15	ST533	—	dog	yes	R	R	R	R	S	S
FMV5338/13	2013	CTX-M-15	ST131:H4-B2-O25b	CC131	dog	yes	I	R	S	R	R	R
FMV58/2013	2013	CTX-M-1-type	ST131:H4-B2-O25b	CC131	cat	yes	S	R	R	R	S	R
FMV121/2014RE	2014	CTX-M-1-type	ST539	—	dog	yes	S	R	R	R	S	R

AMC, amoxicillin/clavulanate; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible.
[a]Also harbours bla_{CMY-2}.
[b]Refer to Figure S1 to see the new ST allelic profile.

Nevertheless, from 1999/2006 to 2007/2014, *E. coli* showed a 3-fold increase in amoxicillin/clavulanate resistance and a 4-fold increase in 3GC resistance. *Proteus* spp. had an even higher increase in resistance, showing a 5- and 9-fold increase in amoxicillin/clavulanate and 3GC resistance, respectively (Table S4). *Proteus* spp. and *E. coli* also had a significant increase in gentamicin resistance (Tables S3 and S4). Detection of MDR Enterobacteriaceae, *E. coli* and *Proteus* spp. increased significantly over time (Tables S3 and S4 and Figure 1). Most MDR Enterobacteriaceae (excluding *Enterobacter* spp.) were susceptible to at least one antimicrobial (Table S5). Gentamicin was the antimicrobial to which most MDR Enterobacteriaceae (excluding *Enterobacter* spp.) were susceptible (46.2%, CI 36.1%–56.4%). On the contrary, MDR Enterobacteriaceae were seldom susceptible to fluoroquinolones (9.7%, CI 3.7%–15.7%) (Table S5). Furthermore, among MDR *E. coli*, only 19.35% (CI 9.5%–29.2%) were susceptible to tetracycline.

A total of 33 *E. coli* and 9 *P. mirabilis* that were 3GC-resistant were recovered and screened for the presence of ESBL and AmpC genes. The first 3GC-resistant *E. coli* isolate was detected in 1999, yet none of the tested genes were detected. Another resistance mechanism may be involved. In total, only seven 3GC-resistant *E. coli* were negative for all tested genes, including $bla_{TEM-type}$ and $bla_{SHV-type}$ ESBLs. *E. coli* 3GC resistance was mainly related to the presence of $bla_{CTX-M-15}$ and bla_{CMY-2} (Tables 1 and 2). The first CTX-M-producing *E. coli* was detected in 2004 and belonged to the O25b:H4-B2-ST131 clonal lineage (Table 1). Besides O25b:H4-B2-ST131, CTX-M-producing *E. coli* were frequently found to belong to clonal complex (CC) 23, including a novel ST described here (Table 1 and Figure S1). From 2010 onwards, an increase in 3GC-resistant *E. coli* ST648 harbouring bla_{CMY-2} was observed (Table 2). 3GC-resistant *P. mirabilis* was first detected in 2004. All isolates with this phenotype were bla_{CMY-2} positive, except one *P. mirabilis* from 2007 possessing bla_{DHA-1} (Table S6).

Regarding *Staphylococcus* spp., 9.2% (CI 4.0%–14.4%, $n = 11/119$) of *Staphylococcus* spp. were methicillin resistant and were found to harbour the *mecA* gene. Overall only resistance to fluoroquinolones was significantly higher in the second time period (2007–14; $P = 0.0189$) (Table S4). However, if *S. pseudintermedius* are analysed alone, a significant increase in methicillin (14.8%, CI 1.4%–28.2%, in 2007–14; $P = 0.0069$) and gentamicin (17.9%, CI 3.7%–32.0%, in 2007–14; $P = 0.0099$) resistance was also detected. All MDR *Staphylococcus* spp. were associated with the presence of the *mecA* gene. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP; $n = 4$) and methicillin-resistant *S. epidermidis* (MRSE; $n = 4$) were the most common methicillin-resistant *Staphylococcus* species, followed by MRSA ($n = 2$) and methicillin-resistant *Staphylococcus lentus* ($n = 1$). Although uncommon, *S. epidermidis* showed a high frequency of methicillin resistance ($n = 4/6$). MRSP showed resistance to all the tested antimicrobials, except two that were susceptible to tetracycline. Thus all MRSP were considered MDR. All methicillin-resistant *Staphylococcus* spp. but one were isolated from cats. The 11 methicillin-resistant *Staphylococcus* spp. found in this study were fully characterized elsewhere.[8] Briefly, MRSP belonged to ST71-II-III ($n = 3$) and ST196-V ($n = 1$); MRSE belonged to CC 5 (ST2-nt, ST20-nt, ST23-IV, ST35-nt) and MRSA to CC5 (ST5-t311-VI, ST105-t002-II).

Enterococcus spp. showed very high tetracycline (75.8%, CI 65.2%–86.5%, $n = 47/62$) and fluoroquinolone (56.4%, CI 44.1%–68.8%, $n = 35/62$) resistance. Ampicillin resistance in *Enterococcus* spp. (12.1%, CI 4.2%–20.0%, $n = 8/66$) was lower, though if only *E. faecium* were considered, almost all were ampicillin resistant ($n = 8/9$). HLGR was detected in 15.2% (CI 6.1%–24.4%, $n = 9/59$) of the tested *Enterococcus* spp. and was mostly found in *E. faecalis* harbouring *aac(6′)-Ieaph(2″)-Ia* (Table S7). HLGR *E. faecalis* belonged to known sequence types, including one isolate from ST6-CC6 (former CC2) (Table S7). All ampicillin-resistant and/or HLGR *E. faecium* belonged to CC17 (Table S7), including two novel STs (ST1282 and ST1283) identified in this study (Figure S2).

Table 2. AmpC-producing *E. coli* clonal lineages

Isolate	Year	β-Lactamase	Clonal lineage	CC	Animal	MDR	AMC	3GCs	SXT	FQs	GEN	TET
FMV434/00	2000	CMY-2	ST1775	—	dog	yes	R	R	R	S	S	R
FMV1953/01	2001	CMY-2	ST57	CC350	dog	yes	R	R	R	I	I	R
FMV203/03	2003	CMY-2	ST405	CC405	dog	yes	R	R	S	R	S	R
FMV6346/05	2005	CMY-2	ST539	—	cat	yes	R	R	S	R	R	R
FMV3389/06	2006	CMY-2	ST354	CC354	dog	yes	R	R	R	R	R	R
FMV1952/10[a]	2010	CMY-2	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV25/2011	2011	CMY-2	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV29/2011	2011	CMY-2	ST648	CC648	dog	yes	R	R	S	R	R	R
FMV469/13	2013	CMY-2	ST648	CC648	dog	yes	R	R	R	R	R	R
FMV1389/13	2013	CMY-2	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV4479/13[a]	2013	CMY-2	ST533	—	dog	yes	R	R	R	R	S	S
FMV55/2013	2013	CMY-2	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV546/14	2014	CMY-2	ST648	CC648	cat	yes	R	R	R	R	R	R
FMV966/14	2014	CMY-2	ST648	CC648	dog	yes	R	R	R	R	S	R
FMV1549/14	2014	CMY-2	ST648	CC648	cat	yes	R	R	S	R	S	R
FMV43/2014	2014	CMY-2	ST648	CC648	cat	yes	R	R	S	R	S	R

AMC, amoxicillin/clavulanate; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible.
[a]Also harbours bla_{CTX-M}.

Discussion

As seen in other studies, *E. coli* was the most frequently isolated bacterium in dogs and cats with UTI.[1,4] Overall, Enterobacteriaceae caused more than half of the UTIs in dogs and cats; therefore, given that β-lactams are among the most important antimicrobials nowadays,[10] the great increase detected in this study in Enterobacteriaceae resistance to amoxicillin/clavulanate and 3GCs is worrisome (Figure 1 and Table S4).

3GCs are considered highest-priority critically important antimicrobials for humans,[10] and resistance is frequently associated with the production of β-lactamases.[3] The increase in 3GC resistance observed in this study was frequently associated with the presence of $bla_{CTX-M-15}$ and bla_{CMY-2} in *E. coli* and bla_{CMY-2} in *P. mirabilis* (Tables 1 and 2 and Table S6).

ESBL CTX-M-15 is distributed worldwide in *E. coli*.[31] Moreover, *E. coli* O25b:H4-B2-ST131 is a widespread human uropathogenic clonal lineage that frequently harbours $bla_{CTX-M-15}$[32] and has been previously described in dog and cat faecal samples (as a commensal),[12] and also causes UTI.[6] Therefore the detection of *E. coli* O25b:H4-B2-ST131 in this study came as no surprise. Furthermore, *E. coli* CC23 has been described in humans with UTI in the community[33] and has also been shown to be a common CC among CTX-M-producing *E. coli*.[34]

This study shows an increase in the detection of CMY-2-producing *E. coli* and *Proteus* spp. over time. Although less frequently reported than bla_{CTX-M} in previously published data,[6,35] this increase in bla_{CMY-2} should not be neglected since this enzyme shows stronger β-lactamase activity[36] and may in the future become more prevalent. Furthermore, bla_{CMY-2}-carrying Enterobacteriaceae may exhibit resistance to carbapenems in the absence of carbapenemases, owing to the presence of other resistance mechanisms such as porin defiency,[37] which further highlights their clinical relevance.

In the present study, the first CMY-2-producing *E. coli* was detected in 2000, yet it was from 2010 onwards that the *E. coli* CMY-2-producing ST648 clonal lineage was increasingly detected (Table 2). *E. coli* ST648 has been described in human infections harbouring several β-lactamases, such as ESBL and carbapenemases.[31,38] Nevertheless, both in humans and companion animals, the ST648 *E. coli* clonal lineage has mostly been described harbouring bla_{CTX-M} genes.[38,39] The significant increase in ST648 CMY-2-producing *E. coli* observed in this study in companion animals with UTI may point to the possible expansion of a bla_{CMY-2}-producing MDR ST648 clonal lineage, though more studies are needed to clarify the clonal relatedness between these isolates. However, a few studies have also found a high frequency of ST648 CMY-2-producing *E. coli* in faecal samples and specimens from infections of companion animals.[40,41] Although only detected once in this study, *E. coli* ST405 also belongs to a highly successful clonal lineage that causes human infection.[38] Dogs and cats with UTI are, therefore, shown in this study to be infected with 3GC-resistant *E. coli* belonging to clonal lineages of great importance to humans. Furthermore, CTX-M- and CMY-producing *E. coli* and *P. mirabilis* were also found to be MDR, thus increasing the relevance of these findings.

Additionally, Enterobacteriaceae from this study showed a significant increase in resistance to all the antimicrobials commonly used in the treatment of dogs and cats with UTI (Figure 1). Together with the significant increase in detection of MDR Enterobacteriaceae over time, these results point to growing therapeutic limitations in veterinary medicine that in the future may lead to an increasing need to prescribe antimicrobials originally intended for human use.

ESBL- and AmpC-producing *E. coli* presented MDR susceptibility phenotypes with limited therapeutic options (Tables 1 and 2). A study in humans showed that the use of amoxicillin/clavulanate for the treatment of UTIs caused by ESBL-producing *E. coli* may be suitable if the isolate is fully susceptible to this antimicrobial.[42] Although most ESBL-producing *E. coli* from this study displayed intermediate resistance to amoxicillin/clavulanate, similar studies should be conducted in veterinary medicine to evaluate its effectiveness in the treatment of MDR ESBL-producing Enterobacteriaceae.

MDR Enterobacteriaceae were more often susceptible to gentamicin; hence, although sometimes impractical, the use of this antimicrobial should be considered for the treatment of UTIs caused by MDR Enterobacteriaceae in dogs and cats (Table S5).

Staphylococcus spp. were the second most frequently isolated bacteria; however, the identified *Staphylococcus* species varied significantly between dogs and cats (Table S2). The high frequency of *S. felis* in cats with UTI is in agreement with a previous study conducted in Australia, in which the authors associated the presence of *S. felis* with clinical signs of lower urinary tract disease in cats.[4]

The significant increase in the detection of MDR MRSP over time is a concerning finding since it creates major therapeutic limitations. The MRSP detected in this study belonged mainly to ST71-II-III, which is known to be one of the most disseminated clonal lineages in dogs and cats in Europe.[43,44] Although rarely, human infection by MRSP ST71-II-III has already been described, thus highlighting its zoonotic potential.[45]

As seen in reports on humans, *S. epidermidis* showed a high frequency of methicillin resistance[46] and all belonged to STs also found in humans.[47] The two MRSA isolated in this study belonged to *S. aureus* CC5, which is frequently associated with human hospital-acquired MRSA.[48] Furthermore, both MRSA STs have been reported in humans from Portugal.[49,50] Interestingly, in this study, the *mec*A gene was mainly detected in *Staphylococcus* spp. isolated from cats. As this was a retrospective study, it was not possible to obtain information on the possible source of these infections. Nevertheless, the detection of MRSA and MRSE in dogs and cats with UTI is a public health issue since companion animals will have a role in dissemination of these bacteria to the household and public environment.

Ampicillin resistance and HLGR in *Enterococcus* spp. strongly limit the therapeutic options against enterococcal infections.[51] Although in this study ampicillin-resistant and/or HLGR *Enterococcus* spp. were uncommon, some belonged to high-risk CCs associated with hospital-acquired infections in humans, such as *E. faecalis* CC6 (formerly CC2) and *E. faecium* CC17 (Table S7).[52,53] HLGR was mostly detected in *E. faecalis* and was caused by the presence of a bifunctional enzyme that is known to also confer resistance to a wide range of aminoglycosides.[51] *E. faecalis* ST6 (CC6) has been previously described in hospitalized patients from Portugal but also in samples from pigs and from hospital waste waters.[54] As in this study, the *E. faecalis* ST16 is a clonal lineage that has been found to frequently harbour the bifunctional enzyme.[29,55] Also, *E. faecalis* ST16 has been previously detected in healthy and hospitalized humans as well as in animals.[29,54,55]

E. faecium isolates were less frequent than those of *E. faecalis*, but were more commonly ampicillin resistant (Table S7). A previous study has pointed to healthy dogs as reservoirs of ampicillin-resistant *E. faecium* CC17 including ST19.[56] Besides belonging to CC17, the *E. faecium* ST19 and ST440 strains isolated in this study are also noteworthy for being simultaneously ampicillin resistant and HLGR.

Enterobacteriaceae and *Staphylococcus* spp. together caused around 79% of the UTIs in dogs and cats. Thus, the increase in fluoroquinolone resistance in both groups of bacteria is of great relevance. Furthermore, *Enterococcus* spp. also showed high levels of resistance to this antimicrobial class. Bearing in mind fluoroquinolones are considered highest-priority critically important antimicrobials for humans,[10] and of great importance in the treatment of pyelonephritis in companion animals,[2] judicious use of these antimicrobials should be pursued.

The fact that this study relied on samples submitted to the laboratory based on clinical judgement could be considered a bias towards resistance because urine cultures from complicated UTIs may be requested more frequently than from simple uncomplicated UTIs.[1] However, if present, this bias was constant throughout the study time frame and therefore the increase in antimicrobial resistance is unequivocal.

Only in recent years has the EMA started to publish data about antimicrobial sales for companion animals. Nevertheless, the high β-lactam and fluoroquinolone resistance frequencies detected in this study could be expected since these are the first and second most sold antimicrobials for companion animals in Portugal.[57] The high frequency of tick-borne diseases in Portugal that require the use of doxycycline in companion animals could have contributed to the high tetracycline resistance seen in this study. Also, the increase in MDR Enterobacteriaceae may, to some extent, explain the increasing resistance trends observed for all the tested antimicrobials.

The limited access to complete data about the epidemiological and clinical history of the dogs and cats infected with high-risk clonal lineages was also a limitation of this study. Nevertheless, since most of these animals were treated at home by their owners, the role of dogs and cats with UTI in the spread of resistant bacteria into the environment should be considered. Furthermore, it has been shown that companion animals may share uropathogenic bacteria with their household members,[12] and therefore the role of dogs and cats as reservoirs of high-risk clonal lineages is a concern to veterinary professionals and owners.

This study showed that bacteria causing UTI in dogs and cats, especially Enterobacteriaceae, are increasingly resistant to the antimicrobials most widely used in UTI treatment. These bacteria were found to harbour clinically relevant antimicrobial resistance mechanisms and simultaneously belong to high-risk clonal lineages, namely 3GC-resistant *E. coli* O25b:H4-B2-ST131, CC23 and ST648, MRSA CC5, MRSE CC5, HLGR *E. faecalis* CC6, and ampicillin/HLGR *E. faecium* CC17. Therefore, when such resistance mechanisms are suspected based on susceptibility testing, veterinary professionals and owners should be advised to take measures in order to reduce the spread of these bacteria, such as strict hand washing, cleaning of the animals' living environment as well as adequate faecal disposal. Also, longitudinal studies on the faecal carriage of these resistant high-risk clonal lineages during and after UTI should be conducted to assess the time of carriage and evaluate the extent and duration of the infection control measures that should be taken.

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Transparency declarations

None to declare.

Supplementary data

Tables S1–S7 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Hall JL, Holmes MA, Baine SJ. Prevalence and antimicrobial resistance of canine urinary tract pathogens. *Vet Rec* 2013; **173**: 549.

2 Weese S, Blondeau JM, Boothe D *et al*. Antimicrobial use guidelines for treatment of urinary tract disease in dogs and cats: Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. *Vet Med Int* 2011; **2011**: 263768.

3 ECDC. *Antimicrobial Resistance Surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)*. Stockholm: ECDC, 2015.

4 Litster A, Moss SM, Honnery M *et al*. Prevalence of bacterial species in cats with clinical signs of lower urinary tract disease: recognition of *Staphylococcus felis* as a possible feline urinary tract pathogen. *Vet Microbiol* 2007; **121**: 182–8.

5 Féria C, Ferreira E, Correia JD *et al*. Patterns and mechanisms of resistance to β-lactams and β-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. *J Antimicrob Chemother* 2002; **49**: 77–85.

6 Ewers C, Grobbel M, Stamm I *et al*. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-β-lactamase-producing *Escherichia coli* among companion animals. *J Antimicrob Chemother* 2010; **65**: 651–60.

7 Wagner S, Gally DL, Argyle SA. Multidrug-resistant *Escherichia coli* from canine urinary tract infections tend to have commensal phylotypes, lower prevalence of virulence determinants and *ampC*-replicons. *Vet Microbiol* 2014; **169**: 171–8.

8 Couto N, Monchique C, Belas A *et al*. Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period. *J Antimicrob Chemother* 2016; **71**: 1479–87.

9 Jackson CR, Fedorka-Cray PJ, Davis JA *et al*. Mechanisms of antimicrobial resistance and genetic relatedness among enterococci isolated from dogs and cats in the United States. *J Appl Microbiol* 2010; **108**: 2171–9.

10 WHO. *Critically Important Antimicrobials for Human Medicine—5th Revision*. 2017. http://apps.who.int/iris/bitstream/10665/255027/1/9789241512220-eng.pdf.

11 Pomba C, Rantala M, Greko C *et al*. Public health risk of antimicrobial resistance transfer from companion animals. *J Antimicrob Chemother* 2017; **72**: 957–68.

12 Johnson JR, Miller S, Johnston B *et al*. Sharing of *Escherichia coli* sequence type ST131 and other multidrug-resistant and urovirulent *E. coli* strains among dogs and cats within a household. *J Clin Microbiol* 2009; **47**: 3721–5.

13 Bartges JW. Diagnosis of urinary tract infections. *Vet Clin North Am Small Anim Pract* 2004; **34**: 923–33.

14 Doumith M, Day MJ, Hope R *et al*. Improved multiplex PCR strategy for rapid assignment of the four major *Escherichia coli* phylogenetic groups. *J Clin Microbiol* 2012; **50**: 3108–10.

15 Kovtunovych GL, Lytvynenko T, Negrutska VV *et al*. A PCR-mediated method for discrimination of *Klebsiella oxytoca* between closely related bacteria in environmental and clinical specimens. *Biopolym Cell* 2003; **19**: 520–4.

16 Padmavathy B, Vinoth Kumar R, Patel A *et al*. Rapid and sensitive detection of major uropathogens in a single-pot multiplex PCR assay. *Curr Microbiol* 2012; **65**: 44–53.

17 Stankowska D, Kwinkowski M, Kaca W. Quantification of *Proteus mirabilis* virulence factors and modulation by acylated homoserine lactones. *J Microbiol Immunol Infect* 2008; **41**: 243–53.

18 Woodford N, Egelton CM, Morrison D. Comparison of PCR with phenotypic methods for the speciation of enterococci. *Adv Exp Med Biol* 1997; **418**: 405–8.

19 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals—Fourth Edition: Approved Standard VET01-A4*. CLSI, Wayne, PA, USA, 2013.

20 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Second Informational Supplement VET01-S2*. CLSI, Wayne, PA, USA, 2013.

21 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Sixth Informational Supplement M100-S26*. CLSI, Wayne, PA, USA, 2016.

22 Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; **40**: 2153–62.

23 Guessennd N, Bremont S, Gbonon V *et al*. Résistance aux quinolones de type qnr chez les entérobactéries productrices de bêta-lactamases à spectre élargi à Abidjan en Côte d'Ivoire. *Pathol Biol (Paris)* 2008; **56**: 439–46.

24 Belas A, Salazar AS, Telo da Gama L *et al*. Risk factors for faecal colonisation with *Escherichia coli* producing extended-spectrum and plasmid-mediated AmpC β-lactamases in dogs. *Vet Rec* 2014; **175**: 202.

25 Kao SJ, You I, Clewell DB *et al*. Detection of the high-level aminoglycoside resistance gene *aph*(2")-Ib in *Enterococcus faecium*. *Antimicrob Agents Chemother* 2000; **44**: 2876–9.

26 Johnson J, Clermont O, Johnstone B *et al*. Rapid and specific detection, molecular epidemiology, and experimental virulence of the O16 subgroup within *Escherichia coli* sequence type 131. *J Clin Microbiol* 2014; **52**: 1358–65.

27 Wirth T, Falush D, Lan R *et al*. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 2006; **60**: 1136–51.

28 Homan WL, Tribe D, Poznanski S *et al*. Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 2002; **40**: 1963–71.

29 Ruiz-Garbajosa P, Bonten MJ, Robinson DA *et al*. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J Clin Microbiol* 2006; **44**: 2220–8.

30 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

31 Ewers C, Bethe A, Semmler T *et al*. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; **18**: 646–55.

32 Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V *et al*. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. *J Antimicrob Chemother* 2008; **61**: 273–81.

33 Lau SH, Reddy S, Cheesbrough J *et al*. Major uropathogenic *Escherichia coli* strain isolated in the northwest of England identified by multilocus sequence typing. *J Clin Microbiol* 2008; **46**: 1076–80.

34 Brisse S, Diancourt L, Laouénan C *et al*. Phylogenetic distribution of CTX-M- and non-extended-spectrum-β-lactamase-producing *Escherichia coli* isolates: group B2 isolates, except clone ST131, rarely produce CTX-M enzymes. *J Clin Microbiol* 2012; **50**: 2974–81.

35 Aragón LM, Mirelis B, Miró E *et al*. Increase in β-lactam-resistant *Proteus mirabilis* strains due to CTX-M- and CMY-type as well as new VEB- and inhibitor-resistant TEM-type β-lactamases. *J Antimicrob Chemother* 2008; **61**: 1029–32.

36 Jacoby GA, Amp C. β-lactamases. *Clin Microbiol Rev* 2009; **22**: 161–82.

37 Chia JH, Siu LK, Su LH *et al*. Emergence of carbapenem-resistant *Escherichia coli* in Taiwan: resistance due to combined CMY-2 production and porin deficiency. *J Chemother* 2009; **21**: 621–6.

38 Pitout JDD. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. *Front Microbiol* 2012; **3**: 9.

39 Ewers C, Bethe A, Stamm I *et al*. CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? *J Antimicrob Chemother* 2014; **69**: 1224–30.

40 Tamang MD, Nam HM, Jang GC *et al*. Molecular characterization of extended-spectrum-β-lactamase-producing and plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* isolated from stray dogs in South Korea. *Antimicrob Agents Chemother* 2012; **56**: 2705–12.

41 Liu X, Thungrat K, Boothe DM. Occurrence of OXA-48 carbapenemase and other β-lactamase genes in ESBL-producing multidrug resistant *Escherichia coli* from dogs and cats in the United States, 2009–2013. *Front Microbiol* 2016; **7**: 1057.

42 Beytur A, Yakupogullari Y, Oguz F *et al*. Oral amoxicillin-clavulanic acid treatment in urinary tract infections caused by extended-spectrum β-lactamase-producing organisms. *Jundishapur J Microbiol* 2015; **8**: e13792.

43 Kadlec K, Schwarz S, Perreten V *et al*. Molecular analysis of methicillin-resistant *Staphylococcus pseudintermedius* of feline origin from different European countries and North America. *J Antimicrob Chemother* 2010; **65**: 1826–8.

44 Perreten V, Kadlec K, Schwarz S *et al*. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. *J Antimicrob Chemother* 2010; **65**: 1145–54.

45 Stegmann R, Burnens A, Maranta CA *et al*. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius* ST71. *J Antimicrob Chemother* 2010; **65**: 2047–8.

46 Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. *Clin Microbiol Rev* 2014; **27**: 870–926.

47 Rolo J, de Lencastre H, Miragaia M. Strategies of adaptation of *Staphylococcus epidermidis* to hospital and community: amplification and diversification of SCC*mec*. *J Antimicrob Chemother* 2012; **67**: 1333–41.

48 Aires-de-Sousa M. Methicillin-resistant *Staphylococcus aureus* among animals: current overview. *Clin Microbiol Infect* 2017; **23**: 373–80.

49 Conceição T, Tavares A, Miragaia M *et al*. Prevalence and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Atlantic Azores islands: predominance of SCC*mec* types IV, V and VI. *Eur J Clin Microbiol Infect Dis* 2010; **29**: 543–50.

50 Espadinha D, Faria NA, Miragaia M *et al*. Extensive dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) between the hospital and the community in a country with a high prevalence of nosocomial MRSA. *PLoS One* 2013; **8**: e59960.

51 Chow JW. Aminoglycoside resistance in enterococci. *Clin Infect Dis* 2000; **31**: 586–9.

52 Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006; **9**: 454–60.

53 Kuch A, Willems RJ, Werner G *et al*. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J Antimicrob Chemother* 2012; **67**: 551–8.

54 Freitas AR, Novais C, Ruiz-Garbajosa P *et al*. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal. *J Antimicrob Chemother* 2009; **63**: 1104–11.

55 Larsen J, Schønheyder HC, Lester CH *et al*. Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* 2010; **16**: 682–4.

56 Damborg P, Top J, Hendrickx AP *et al*. Dogs are a reservoir of ampicillin-resistant *Enterococcus faecium* lineages associated with human infections. *Appl Environ Microbiol* 2009; **75**: 2360–5.

57 EMA. *Sales of Veterinary Antimicrobial Agents in 29 European Countries in* 2014. 2016. http://www.ema.europa.eu/docs/en_GB/document_library/Report/2016/10/WC500214217.pdf.