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SHORT  
COMMUNICATION



# Detection of *bla*<sub>CTX-M</sub> genes in ESBL-producing *Klebsiella* isolates from animals in Croatia

SELMA PINTARIĆ<sup>1</sup>, ZRINKA ŠTRITOF<sup>1\*</sup> ,  
VESNA MOJČEC PERKO<sup>1</sup>, ANDREA TUMPA<sup>2</sup>,  
MARIJA CVETNIĆ<sup>1</sup> and LANA HADŽIĆ<sup>3</sup>

<sup>1</sup> Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, Zagreb, Croatia

<sup>2</sup> Department of Chemistry and Biochemistry, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, Zagreb, Croatia

<sup>3</sup> Laboratory for Food, Water and Animal Feed Microbiology, PI 'Veterinary Institute', Bihać, Bosnia and Herzegovina

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## ABSTRACT

This study investigated the frequency of third-generation cephalosporin resistance in *Klebsiella* spp. isolated from animals in Croatia and the presence of *bla*<sub>CTX-M</sub> genes. A total of 711 enteric bacteria were isolated from clinical samples, and *Klebsiella* spp. isolates accounted for 6.9% ( $n = 49$ ). Thirteen *Klebsiella* isolates (26.5%) were ESBL producers, nine isolates from the *Klebsiella pneumoniae* species complex (69.2%), and four (30.8%) *Klebsiella oxytoca* isolates. All carried the *bla*<sub>CTX-M-15</sub> gene, and antimicrobial susceptibility testing revealed them as multidrug resistant. All were resistant to all tested cephalosporins, fluoroquinolones, aminoglycosides and aztreonam, 92.3% showed resistance to tetracycline, 84.6% to trimethoprim-sulfamethoxazole and 69.2% to nitrofurantoin. No isolate showed resistance to imipenem and meropenem. It can be concluded that ESBL-producing *Klebsiella* expressing the *bla*<sub>CTX-M</sub> gene are not rare among *Klebsiella* isolates from animals in Croatia.

## KEYWORDS

*Klebsiella* spp., ESBL, *bla*<sub>CTX-M</sub>, animals, multidrug resistance

The occurrence of infections caused by extended-spectrum beta-lactamase (ESBL)-producing *Enterobacterales* is increasing every year, mainly because of indiscriminate antimicrobial therapy. ESBLs are a group of enzymes that share the ability to hydrolyse the beta-lactam ring, compromising the efficacy of all beta-lactams except cephamycins and carbapenems, but are generally susceptible to  $\beta$ -lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. The genes encoding ESBLs, the *bla*<sub>ESBL</sub> genes, are generally located on highly mobile plasmids that may also simultaneously carry genes conferring resistance to several other unrelated classes of antimicrobials such as quinolones and aminoglycosides. Therefore, options for the antibiotic treatment of ESBL-producing organisms are very limited.

ESBLs can be classified into three main types, namely TEM, SHV and CTX-M, of which the CTX-M type has been increasingly reported worldwide, making this group of enzymes the most common ESBL type (Castanheira et al., 2021).

The presence of ESBL producers in animals is a potential public health risk because animals can serve as reservoirs of resistant bacteria (Marques et al., 2019). There are almost no data on resistance, especially on resistance mechanisms, in *Klebsiella* isolates from animals in Croatia, while human *Klebsiella* spp. and other *Enterobacterales* isolates positive for CTX-M beta-lactamase are widely distributed (Bedenić et al., 2010; Krilanović et al., 2020). The aim of this study was to determine the prevalence and type of CTX-M beta-lactamases in *Klebsiella* strains isolated from animals in Croatia.

\*Corresponding author.

E-mail: zstritof@vef.unizg.hr

Between January 2016 and October 2021, 711 *Enterobacteriales* isolates from animals in Croatia were collected in the bacteriological laboratory of the Faculty of Veterinary Medicine, University of Zagreb. Most of the isolates (71.2%) were obtained from animals examined at the University's Small Animal Hospital, while 28.8% of the isolates came from animals treated in private veterinary practices in Croatia. A history of antimicrobial therapy was not available.

All Gram-negative, lactose-positive, and oxidase-negative bacilli with positive citrate utilisation test were screened for ESBL production using a cefpodoxime disk, as recommended by the EUCAST (EUCAST, 2017).

Phenotypic confirmation of ESBL production was performed using a commercially available combined disk test for the detection of AmpC and/or ESBL production (MastGroup Ltd., UK) according to the manufacturer's instructions. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.

Isolates that were phenotypically positive for ESBL production were identified using the ID32E identification system (bioMérieux, France). Isolates identified as *Klebsiella* spp. were used for further analysis. Repeated isolates from the same animal were excluded. Isolates were tested for susceptibility to 15 antimicrobials using the standard disk diffusion method on Mueller-Hinton agar (Bio-Rad, France). The diameters of the zones of inhibition for most antimicrobials tested were interpreted according to EUCAST (EUCAST, 2023). The inhibition zone diameters for antimicrobials without susceptibility breakpoints (tetracycline), with different concentrations (cefotaxime, ceftazidime, and nitrofurantoin), and for antimicrobials with veterinary-specific breakpoints (enrofloxacin) were interpreted according to the criteria recommended in CLSI documents VET01S and M100S (CLSI, 2015; CLSI, 2018). The strain *E. coli* ATCC 25922 was used as a quality control strain. The list of antimicrobials and zone diameter interpretive criteria are summarised in Table 1.

Isolates with inhibition zone diameters for meropenem less than 28 mm were evaluated for carbapenemase production using the modified Hodge Test (MHT).

Genomic DNA was isolated from overnight cultures grown on Columbia agar (MastGroup Ltd., UK) using 2% Chelex-100 solution (Bio-Rad, USA) (Matanović et al., 2012). Detection of the *bla*<sub>CTX-M</sub> gene was performed by PCR using primers and conditions as described previously (Pagani et al., 2003).

Additional PCR was performed for *bla*<sub>CTX-M</sub>-positive isolates to generate a 948-bp PCR gene fragment for sequencing using primers described by Ahmed et al. (2007). The following reaction parameters were used: initial denaturation at 95 °C for 120 s followed by denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and elongation at 72 °C for 60 s, repeated for 30 cycles and a final extension at 72 °C for 5 min.

PCR reactions were performed in a 20-μl reaction mixture containing 0.5 μl aliquot of isolated DNA diluted 1:10, 10 μl 2× EmeraldAmp GT PCR Master Mix (Takara Bio Inc.), and 1 μl of each primer (10 μM). Reactions were performed in a thermocycler T100 Thermal cycler, Bio-Rad.

PCR products were sequenced by MacroGen Europe, Amsterdam, Netherlands and results were analysed using the Basic Local Alignment Search Tool (BLAST).

The *E. coli* strain positive for *bla*<sub>CTX-M-15</sub> gene, previously characterised and published by Bedenić et al. (2014) was used as a positive control, while *E. coli* ATCC 25922 was used as a negative control.

Of the 711 *Enterobacteriales* isolates, 527 (74.1%) were from dogs, 82 (11.5%) from cats, 35 (4.9%) from horses, 23 (3.2%) from domestic ruminants, 23 (3.2%) from wild animals, 11 (1.6%) from rabbits, 6 (0.9%) from pigs, and 4 (0.6%) from unknown origin. Among them, 49 (6.9%) isolates were identified as *Klebsiella* spp. Of these, 30 (61.2%) were from dogs, 11 (22.4%) from horses, four (8.2%) from

Table 1. List of antimicrobials, their concentrations and zone diameter interpretative standards for *Enterobacteriaceae*

Antimicrobial agent (abbreviation)	Disk content (μg)	Zone diameter interpretive criteria (nearest whole mm)			References
		S	I	R	
cefotaxime (CTX)	30	≥26	23–25	≤22	CLSI (2018)
ceftriaxone (CRO)	30	≥25	22–24	<22	EUCAST (2023)
ceftazidime (CAZ)	30	≥21	18–20	≤17	CLSI (2018)
cefpodoxime (CPD)	10	≥21	–	<21	EUCAST (2023)
cefepime (FEP)	30	≥27	24–26	<24	EUCAST (2023)
imipenem (IPM)	10	≥22	19–21	<19	EUCAST (2023)
meropenem (MEM)	10	≥22	16–21	<16	EUCAST (2023)
aztreonam (ATM)	30	≥26	21–25	<21	EUCAST (2023)
gentamicin (GM)	10	≥17	–	<17	EUCAST (2023)
amikacin (AN)	30	≥18	–	<18	EUCAST (2023)
enrofloxacin (ENR)	5	≥23	17–22	≤16	CLSI (2015)
ciprofloxacin (CIP)	5	≥25	22–24	<22	EUCAST (2023)
tetracycline (TE)	30	≥15	12–14	≤11	CLSI (2018)
trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥14	11–13	<11	EUCAST (2023)
nitrofurantoin (FT)	300	≥17	15–16	≤14	CLSI (2018)

S – susceptible, I – intermediate (CLSI); susceptible, increased exposure (EUCAST), R – resistant.



cats, and four (8.2%) from other animal species. ESBL screening test with cefpodoxime disk and confirmatory phenotypic test revealed 13 (26.5%) ESBL-producing *Klebsiella*, representing 1.8% of the total number of enteric bacteria isolated. Of these, 11 (84.6%) isolates were from animals treated at the University's Small Animal Hospital, while two (15.4%) were from animals treated at private veterinary practices in Croatia. Nine isolates (69.2%) belonged to the *K. pneumoniae* species complex and four (30.8%) to *K. oxytoca*. All ESBL-producing *Klebsiella* isolates were from dogs and were recovered from wounds ( $n = 4$ ; 30.8%), urinary tract infections ( $n = 3$ ; 23.1%), surgical site infections ( $n = 2$ ; 15.4%), placenta ( $n = 1$ ; 7.7%), endometrium ( $n = 1$ ; 7.7%), synovial fluid ( $n = 1$ ; 7.7%), and pleural effusion ( $n = 1$ ; 7.7%).

All ESBL-producing *Klebsiella* isolates were positive for the *bla*<sub>CTX-M</sub> gene, and sequence analysis revealed that sequenced genes shared 99.88–100% homology with the *bla*<sub>CTX-M-15</sub> gene in *Klebsiella pneumoniae* strain C26 (GenBank accession number: OP807071.1).

Antibiotic susceptibility testing revealed that all CTX-M positive isolates were multidrug resistant and were resistant to all tested cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, cefepime), fluoroquinolones (enrofloxacin, ciprofloxacin), aminoglycosides (gentamicin, amikacin), and aztreonam. Twelve isolates (92.3%) were resistant to tetracycline and one isolate (7.7%) was susceptible. Trimethoprim-sulfamethoxazole showed similar activity with 11 (84.6%) resistant isolates, one (7.7%) susceptible isolate and one (7.7%) isolate was categorised as 'susceptible, increased exposure'. Nine isolates (69.2%) were resistant to nitrofurantoin, three (23.1%) were susceptible and one (7.7%) was categorised as 'susceptible, increased exposure'. All isolates were susceptible to imipenem. Five (38.5%) isolates were susceptible to meropenem, and the remainder (61.5%) were categorised as 'susceptible, increased exposure'. All isolates had an inhibition zone for meropenem of less than 28 mm and were negative for carbapenemase production in the modified Hodge Test. The identification results of each isolate, their antimicrobial resistance profile, and their AmpC activity can be found in Table 2.

Although the frequency of ESBL-positive *Klebsiella* strains among enteric bacteria in this study was quite low (1.8%), the proportion of *Klebsiella* isolates resistant to third-generation cephalosporins among *Klebsiella* isolates is of concern (13/49; 26.5%). A similar prevalence (20.4%) of ESBL producers among *Klebsiella* isolates has been reported in dogs in Italy (Donati et al., 2014). Moreover, in 2020, more than one-third of human *K. pneumoniae* isolates (33.9%) were resistant to third-generation cephalosporins, and most of them (21.0%) had combined resistance to fluoroquinolones, aminoglycosides, and third-generation cephalosporins (WHO/ECDC, 2022).

Furthermore, the detection of the *bla*<sub>CTX-M-15</sub> gene in our isolates is in accordance with the other studies as this is the most common gene within the CTX-M group reported in human and animal *Klebsiella* isolates worldwide (Ewers et al., 2014; Harada et al., 2016; Zhang et al., 2018; Zogg

Table 2. Identification, antimicrobial resistance profile and AmpC activity of each CTX-M-15 producing *Klebsiella* isolate

Isolate code	Species	Antimicrobial resistance profile	AmpC activity
II-286/16	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
II-525/16	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
II-531/16	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
II-534/16	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
II-563/16	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
II-242/17	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
325/18	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	+
391/21	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	–
257/21	<i>K. pneumoniae</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, SC	–
238/18	<i>K. oxytoca</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, ENR, CIP, TE, SXT, FT	+
632/20	<i>K. oxytoca</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, ENR, CIP, TE	–
767/20	<i>K. oxytoca</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, ENR, CIP, TE, SXT	–
799/20	<i>K. oxytoca</i>	CTX, CRO, CAZ, CPD, FEP, ATM, GM, AN, ENR, CIP, TE	+

SC – species complex; CTX – cefotaxime, CRO – ceftriaxone, CAZ – ceftazidime, CPD – cefpodoxime, FEP – cefepime, IPM – imipenem, MEM – meropenem, ATM – aztreonam, GM – gentamicin, AN – amikacin, ENR – enrofloxacin, CIP – ciprofloxacin, TE – tetracycline, SXT – trimethoprim-sulfamethoxazole, FT – nitrofurantoin.

et al., 2018; Carvalho et al., 2020; Lee et al., 2021). In Croatia, the CTX-M-15 enzyme is also the most common type of beta-lactamase in *K. pneumoniae* strains isolated from urine of non-hospitalised humans (Bedenić et al., 2010). However, molecular typing of isolates is needed to investigate possible clonal relationships between isolates from humans and animals. In addition, ESBL isolates should be examined for the presence of other gene groups (TEM and SHV). We consider the lack of the above as a major limitation of this study.



The presence of MDR ESBL *Klebsiella* spp. detected in dogs in Croatia poses a risk of zoonotic transmission to humans. To minimise the impact of zoonotic pathogens in companion animals on human health, it is strongly advisable to monitor the occurrence of resistant isolates in Croatia in both human and veterinary medicine. Therefore, the authors support the initiative to establish a network to monitor antibiotic resistance in veterinary medicine as part of the One Health concept (Mader et al., 2021).

## REFERENCES

- Ahmed, A. M., Motoi, Y., Sato, M., Maruyama, A., Watanabe, H., Fukumoto, Y. and Shimamoto, T. (2007): Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. *Appl. Environ. Microbiol.* **73**, 6686–6690.
- Bedenić, B., Matanović, K., Mekić, S., Varda-Brkić, D. and Šeol-Martinec, B. (2014): Co-production of CTX-M-15 and CMY-2 in animal *Escherichia coli* isolate from Croatia. in 24th European Congress of Clinical Microbiology and Infectious Diseases. Barcelona, Spain.
- Bedenić, B., Vraneš, J., Bošnjak, Z., Marijan, T., Mlinarić-Džepina, A., Kukovec, T., Knežević, J., Anušić, M., Beader, N., Barl, P., Leskova, V. and Kalenić, S. (2010): Emergence of CTX-M group 1 extended-spectrum  $\beta$ -lactamase-producing *Klebsiella pneumoniae* strains in the community. *Med. Glas.* **7**, 32–39.
- Carvalho, I., Alonso, C. A., Silva, V., Pimenta, P., Cunha, R., Martins, C., Igrejas, G., Torres, C. and Poeta P. (2020): Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolated from healthy and sick dogs in Portugal. *Microb. Drug Resist.* **26**, 709–715.
- Castanheira, M., Simner, P. J. and Bradford, P. A. (2021): Extended-spectrum  $\beta$ -lactamases: an update on their characteristics, epidemiology and detection. *JAC Antimicrob. Resist.* **3**, 3.
- CLSI (2015): Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 3rd ed. CLSI supplement VET01S. Clinical and Laboratory Standards Institute, Wayne, PA.
- CLSI (2018): Performance Standards for Antimicrobial Susceptibility Testing. 28th ed CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.
- Donati, V., Feltrin, F., Hendriksen, R. S., Svendsen, C. A., Cordaro, G., García-Fernández, A., Lorenzetti, S., Lorenzetti, R., Battisti, A. and Franco, A. (2014): Extended-spectrum-beta-lactamases, AmpC beta-lactamases and plasmid mediated quinolone resistance in *Klebsiella* spp. from companion animals in Italy. *PLoS One* **9**(3): e90564.
- EUCAST (2017): The European Committee on Antimicrobial Susceptibility Testing. EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance. Version 2.0. <http://www.eucast.org>.
- EUCAST (2023): The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 13.0. <http://www.eucast.org>.
- Ewers, C., Stamm, I., Pfeifer, Y., Wieler, L. H., Kopp, P. A., Schønning, K., Prenger-Berninghoff, E., Scheufen, S., Stolle, I., Günther, S. and Bethe, A. (2014): Clonal spread of highly successful ST15-CTX-M-15 *Klebsiella pneumoniae* in companion animals and horses. *J. Antimicrob. Chemother.* **69**, 2676–2680.
- Harada, K., Shimizu, T., Mukai, Y., Kuwajima, K., Sato, T., Usui, M., Tamura, Y., Kimura, Y., Miyamoto, T., Tsuyuki, Y., Ohki, A. and Kataoka, Y. (2016): Phenotypic and molecular characterization of antimicrobial resistance in *Klebsiella* spp. isolates from companion animals in Japan: clonal dissemination of multidrug-resistant extended-spectrum  $\beta$ -lactamase-producing *Klebsiella pneumoniae*. *Front. Microbiol.* **29**, 1021.
- Krilanović, M., Tomić-Paradžik, M., Meštrović, T., Beader, N., Herljević, Z., Conzemius, R., Barišić, I., Vraneš, J., Elvedi-Gašparović, V. and Bedenić, B. (2020): Extended-spectrum beta-lactamases and plasmid diversity in urinary isolates of *Escherichia coli* in Croatia: a nation-wide, multicentric, retrospective study. *Folia Microbiol. (Praha)* **65**, 649–667.
- Lee, D., Oh, J. Y., Sum, S. and Park, H. M. (2021): Prevalence and antimicrobial resistance of *Klebsiella* species isolated from clinically ill companion animals. *J. Vet. Sci.* **22**, e17.
- Mader, R., Damborg, P., Amat, J. P., Bengtsson, B., Bourély, C., Broens, E. M., Busani, L., Crespo-Robledo, P., Filippitzi, M. E., Fitzgerald, W., Kaspar, H., Madero, C. M., Norström, M., Nykäsenoja, S., Pedersen, K., Pokludova, L., Urdahl, A. M., Vatopoulos, A., Zafeiridis, C., Madec, J. Y., EU-JAMRAI (2021): Building the European antimicrobial resistance Surveillance network in veterinary medicine (EARS-Vet). *Euro Surveill.* **26**, 2001359.
- Marques, C., Belas, A., Aboim, C., Cavaco-Silva, P., Trigueiro, G., Gama, L. T. and Pombaa, C. (2019): Evidence of sharing of *Klebsiella pneumoniae* strains between healthy companion animals and cohabiting humans. *J. Clin. Microbiol.* **57**, 015377–18.
- Matanović, K., Mekić, S. and Šeol, B. (2012): Antimicrobial susceptibility of *Staphylococcus pseudintermedius* isolated from dogs and cats in Croatia during a six-month period. *Vet. Arhiv* **82**, 505–517.
- Pagani, L., Dell'Amico, E., Migliavacca, R., D'Andrea, M. M., Giacobone, E., Amicosante, G., Romero, E. and Rossolini, G. M. (2003): Multiple CTX-M-type extended-spectrum beta-lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in northern Italy. *J. Clin. Microbiol.* **41**, 4264–4269.
- WHO Regional Office for Europe/European Centre for Disease Prevention and Control (2022): Antimicrobial Resistance Surveillance in Europe 2022–2020 Data. WHO Regional Office for Europe, Copenhagen.
- Zhang, P. L. C., Shen, X., Chalmers, G., Reid-Smith, R. J., Slavic, D., Dick, H. and Boerlin, P. (2018): Prevalence and mechanisms of extended-spectrum cephalosporin resistance in clinical and fecal *Enterobacteriaceae* isolates from dogs in Ontario, Canada. *Vet. Microbiol.* **213**, 82–88.
- Zogg, A. L., Simmen, S., Zurfluh, K., Stephan, R., Schmitt, S. N. and Nüesch-Inderbinen, M. (2018): High prevalence of extended-spectrum  $\beta$ -lactamase producing *Enterobacteriaceae* among clinical isolates from cats and dogs admitted to a veterinary hospital in Switzerland. *Front. Vet. Sci.* **27**, 62.

