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Detection and characterisation of extended-spectrum and plasmid-mediated AmpC β-lactamase produced by *Escherichia coli* isolates found at poultry farms in Bosnia and Herzegovina

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Extended-spectrum β-lactamases (ESBLs) hydrolyse extended-spectrum cephalosporins (ESC) and aztreonam. As ESBL-producing organisms have been identified in food producing animals, the aim of our study was to detect and analyse such *Escherichia coli* isolates from poultry. Antibiotic susceptibility of the isolates was determined with disk-diffusion and broth microdilution methods. ESBLs were detected with the double-disk synergy and inhibitor-based test with clavulanic acid. The transferability of cefotaxime resistance was determined with conjugation experiments, and genes encoding ESBLs, plasmid-mediated AmpC β-lactamases, and quinolone resistance determinants identified by polymerase chain reaction. The study included 108 faecal samples (cloacal swabs) from 25 different poultry farms in the Zenica-Doboj Canton, Bosnia and Herzegovina. Of these, 75 (69.4 %) were positive for *E. coli*, of which 27 were resistant to cefotaxime, amoxicillin, cefazoline, and cefriaxone, and susceptible to imipenem, meropenem, ertapenem, and amikacin. All 27 cefotaxime-resistant isolates were positive in double-disk synergy and combined disk tests. Eighteen isolates transferred cefotaxime resistance to *E. coli* recipient. Twenty-one isolates were positive for the $bla_{\text{CTX-M-1}}$ cluster genes and seven for $bla_{\text{CTX-M-15}}$. Fourteen were positive for the bla_{TEM} genes. The most frequent plasmid incompatibility group was IncFIB, whereas IncFIA and Inc HI1 were present in only a few isolates. Two different sequence types (STs) were identified: ST117 and ST155. The emergence of ESBL-producing *E. coli* in farm animals presents a public health threat, as they can colonise the intestine and cause infections in humans.

KEY WORDS: antimicrobial resistance; chicken; CTX-M; ESBL; public health; ST117; ST155

Extended-spectrum beta-lactamases (ESBLs) produced by bacteria raise public health concern as they mediate the hydrolysis of important antibiotics, including cephalosporins, and aztreonam (1). Recent reports of ESBL-producing bacteria include *Klebsiella pneumoniae*, *Escherichia coli,* other *Enterobacteriaceae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (2–4). Of the three major ESBL families, CTX-M β-lactamases are the most common and, unlike the TEM and SHV families found in hospital pathogens, prevail in community acquired infections (5, 6) and have become a global problem, CTX-M-15 in particular (7).

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As ESBL-encoding genes are located on transferable plasmids, other bacterial species can also get them and become resistant to antibiotics to which they are otherwise susceptible. They are most often found in *K. pneumoniae* (40 %) and *E. coli* (4 %), but their prevalence depends on local epidemiology (8, 9). Unlike ESBLs, plasmid-mediated AmpC β-lactamases are derived from chromosomal β-lactamases of the *Enterobacter*, *Serratia*, *Citrobacter*, *Pseudomonas,* and *Acinetobacter* genera by the escape of the chromosomal gene to the plasmid. They hydrolyse extended-spectrum cephalosporins (ESC), monobactams, and cephamycins and are not susceptible to clavulanic acid, sulbactam, or tazobactam (10).

However, what prompted our research had been recent reports of the global spread of ESBL-producing *E. coli* among livestock (including poultry and pigs) with positive

findings of CTX-M-9, 14, 15, 27, and 32 β-lactamases (11–15). Farm animals were also found to harbour inhibitor-resistant TEM (IRT) β-lactamases IRT-2, 6, and 15 (16).

Considering that reports from Eastern Europe are scarce and there are none from Bosnia and Herzegovina, we carried out this study with the aim to identify and analyse *E. coli* isolates capable of producing ESBLs in poultry raised in our country, as these bacteria can colonise human intestines, especially if the meat is undercooked.

MATERIAL AND METHODS

Bacterial isolates

Between September and October 2019, we took 108 faecal samples (cloacal swabs) from 25 poultry farms located in the Zenica-Doboj Canton (Bosnia and Herzegovina) for diagnostic laboratory investigations, including bacterial isolation and identification. Samples were cultured on blood and MacConkey agar supplemented with 3 mg/L of cefotaxime (Oxoid, Besingstoke, UK) to detect cefotaxime-resistant isolates (17). Using the standard microbiological methods and observing colony morphology and biochemical reactions with indole, Kligler Iron Agar (KIA), citrate agar, phenyl-alanine agar, and urease agar (18) all 27 non-copy isolates were identified as *E. coli*. They were further evaluated at the University Hospital Centre Zagreb, Department for Clinical and Molecular Microbiology using molecular methods.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the 27 cefotaxime-resistant *E. coli* isolates was established with the Kirby-Bauer disk-diffusion and broth microdilution methods according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (19). The same antibiotics were used for both disk-diffusion and broth dilution test, except that some antibiotics such as sulphamethoxazole/trimethoprim, ertapenem, and cefoxitin were tested only with the disk method.

Minimum inhibitory concentrations (MICs) of amoxicillin alone and in combination with clavulanate, piperacillin/tazobactam, cefazoline, extended-spectrum cephalosporins (ESCs; ceftazidime, cefotaxime, and ceftriaxone), cefepime, imipenem, meropenem, gentamicin, or ciprofloxacin were determined with the broth dilution test. The range of tested concentrations was 0.06 to 128 μg/mL. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.

ESBLs were screened for using the double-disk synergy test (DDST) as described elsewhere (20). Briefly, a disk containing amoxicillin with clavulanic acid is placed in the centre of the plate, and disks containing ceftazidime, cefotaxime, ceftriaxone, and cefepime 25 mm apart from the central disk. The test is considered positive if, after overnight incubation at 37 °C, the inhibition zone around cephalosporin disks extends towards the central disk with clavulanic acid.

ESBL production was confirmed with the combined disk test with cephalosporins and clavulanic acid according to CLSI (19). Briefly, the overnight broth culture of the test isolate was diluted to McFarland 0.5 turbidity and swabbed on Mueller-Hinton agar. Disks containing ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), and cefepime (30 μg) were placed on the surface of the agar plate, and 10 μL of clavulanic acid (10 g/L) was dropped on the disks. The control disks contained the same antibiotics but without clavulanate. ESBL production was confirmed if the inhibition zones around ceftazidime, cefotaxime, ceftriaxone, and cefepime disks with clavulanic acid were at least 5 mm wider in diameter than around control disks without it.

Cefotaxime hydrolysis by ESBL was tested with the cephalosporin inactivation method (CIM) as originally described for carbapenem inactivation testing (21). Briefly, disks containing cefotaxime (10 mg) were placed in a heavy suspension of the test strains and the samples were incubated at 37 °C for 2 h. Disks were then taken out and placed on the Mueller-Hinton agar previously inoculated with *E. coli* ATCC 25922 susceptible to cefotaxime. The test was considered positive if there was no inhibition zone, if it was <14 mm in diameter, or if the colonies grew within the inhibition zone.

Four cefoxitin-resistant isolates were tested for plasmid-mediated AmpC β-lactamases using the combined disk test with cephalosporin and 3-aminophenylboronic acid (PBA) disks as described elsewhere (22). Briefly, overnight broth culture of the test isolate was diluted to the 0.5 McFarland turbidity and swabbed on Mueller-Hinton agar. Disks containing ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), and cefepime (30 μg) were placed on the surface of the agar plate and 10 μL of pAmpC-inhibiting PBA was dropped on the disks. The control disks contained the same antibiotics without PBA. The test was considered positive if the inhibition zone around PBA was at least 5 mm longer in diameter than the respective control disk.

Conjugation

The conjugation experiment was performed by "mating" the experimental *E. coli* isolates (donors) with the J65 strain resistant to sodium azide (recipient) according to Elwel and Falkow (23). Overnight broth cultures of donor and recipient strains were mixed in the ratio of 1:2 v/v in Brainheart infusion broth (BHI) and incubated without shaking at 37 °C for another night. Mating mixtures were then placed on combined plates containing cefotaxime (2 mg/L) or ciprofloxacin (1 mg/L) to inhibit the growth of recipient strain and sodium azide (100 mg/L) to inhibit the donor strains. The frequency of conjugation was determined relative to the number of donor cells. The co-transfer of

resistance to non β-lactam antibiotics such as tetracycline, chloramphenicol, sulphamethoxazole/trimethoprim and gentamicin was tested as well.

Molecular detection of resistance genes

The DNA was extracted using the heat lysis protocol as described elsewhere (24). Briefly a heavy suspension of the tested strains prepared in 500 µL of ultrapure water was boiled at 95 °C for 15 min and then spun at 6720 *g* for 2 min. The obtained clear supernatant was used as template DNA for polymerase chain reaction (PCR).

Genes coding for broad and extended-spectrum β-lactamases (bla_{SHV}, bla_{TEM}, bla_{CTX-M}, and bla_{PER-1}), plasmid-mediated AmpC β-lactamases, and quinolone resistance (*qnr*) were detected as described earlier (25–29). Table 1 shows the primers used in this study. The CTX-M β-lactamase cluster was detected with multiplex PCR including five primer pairs: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. For genes coding for CTX-M-8 and CTX-M-25 clusters we used the common reverse primer (30). Amplicons were visualised after electrophoresis in 1 % agarose by staining it with ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). A 100 bp ladder was used as standard to determine the size of the products. The expected size of the amplicons is shown in Table 1. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions (31). DNA was sequenced on both strands by the Eurofins Genomics (Ebersberg, Germany) using the same primers that generated amplified products.

The genetic context of bla_{CTX-M} genes was determined by PCR mapping with forward primer for insertion sequences IS*Ecp1* and IS*26* combined with primer MA-3 (universal reverse primer for bla_{CTX-M} genes) (32). Positive control strains producing TEM-1, TEM-2, SHV-1, and SHV-2 were kindly provided by Professor Adolf Bauernfeind (Max von Pettenkofer Institute, Munich, Germany), and those producing CTX-M-3 and CTX-M-15 by Professor Neil Woodford (Health Protection Agency, London, UK).

Characterisation of plasmids

Plasmids were extracted with the Qiagen Plasmid Mini Kit according to the manufacturer's instructions (33). To

Table 1 Primers for ESBL detection with PCR

Primer designation	Target gene	Sequence	Amplicon size	Ref.
OT 3	TEM	5'-ATG-AGT-ATT- CAA-CAT-TTC-CG-3'	850	25
OT 4	TEM	5'-CCA-ATG-CTT-AAT-CAG-TGA-GG-3'	850	25
SHV-F	SHV	5'-TTC-GCC-TGT-GTA-TTA-TCT-CCC-3	1000	26
SHV-R	SHV	5'-TTA-GCG-TTG-CCA-GTG-YTC-GAT-3'	1000	26
MA-1	CTX-M	5'-SCS-ATG-TGC-AGY-ACC-AGT-AA-3'	550	27
MA-2	CTX-M	5'-CGC-CRA-TAT-GRT-TGG-TGG-TG-3'	550	27
M1-F	CTX-M-1 cluster	5'-AAA-AAT-CAC-TGC-GCC-AGT--TC-3'	415	30
M1-R	CTX-M-1 cluster	5'-TTG-GTG-ACG-ATT-TTA-GCC-GC-3'	415	30
M2-F	CTX-M-2 cluster	5'-CGA-CGC-TAC-CCC-TGC-TAT-T--3'	552	30
M2-R	CTX-M-2 cluster	5'-CCA-GCG-TCA-GAT-TTT-TCA-GG-3'	552	30
M8-F	CTX-M-8 cluster	5-TCG-CGT-TAA-GCG-GAT-GAT-GC	666	30
M9-F	CTX-M-9 cluster	5'-CAA-AGA-GAG-TGC-AAC-GGA-TG	205	30
M9-R	CTX-M-9 cluster	5'ATT-GGA-AAG-CGT-TCA-TCA-CC	205	30
M25-F	CTX-M-25 cluster	5'GCA-CGA-TGA-CAT-TCG-GG	327	30
M9/M25-R	CTX-M-8/25 clusters	5'AAC-CCA-CGA-TGT-GGG-TAG-C		30
IS26-F	IS26	5'-AAA-AAT-GAT-TGA-AAG-GTG-GT-3'		31
IS26-R	IS26	5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3		31
IS*Ecp*-F	IS*Ecp*	5'-AAA-AAT-GAT-TGA-AAG-GTG-GT-3'		31
IS*Ecp*-R	IS*Ecp*	5'-AAT-ACT-ACC-TTG-CTT-TCT-GA-3'		31
QNR A-F	QNR A	5'-ATT-TCT-CAC-GCC-AGG-ATT-TG-3'		29
QNR A-R	QNR A	5'-GAT-CGG-CAA-AGG-TTA-GGT-CA-3'		29
QNR B-F	QNR B	5'-GAT-CGT-GAA-AGC-CAG-AAA-GG		29
QNR B-R	QNR B	5'-ACG-ATG-CCT-GGT-AGT-TGT-CC		29
QNR S-F	QNR S	5'-ACG-ACA-TTC-GTC-AAC-TGC-AA		29
QNR S-R	QNR S	5'-TAA-ATT-GGC-ACC-CTG-TAG-GC		29

identify plasmids coding for ESBLs we relied on PCR-based replicon typing (PBRT) as described by Carattoli et al. (34) updated to identify and distinguish between IncL and IncM plasmids (35). Five multiplex (I, II, III, IV, and V) and two simplex (B and K) reactions were used to determine the incompatibility group based on the size of the product after gel electrophoresis and staining with ethidium bromide. Plasmid extractions from donor and transconjugant strains were subjected to PCR for detection of bla_{ESBL} genes in order to determine their location. Positive control strains were kindly provided by Dr Alessandra Carattoli (Istituto Superiore di Sanità, Rome, Italy).

Genotyping

Two randomly selected isolates were genotyped with multilocus sequence typing (MLST) as described by Wirth et al. (36). Seven housekeeping genes were amplified with PCR, namely *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* (37).

PCR products were detected with agarose gel electrophoresis and then purified and sequenced using the Eurofins service. The obtained sequences were deposited by above mentioned online service to obtain sequence types (STs).

RESULTS

Of the 108 cloacal swabs taken from poultry farms, 75 (69.4 %) had *E. coli*, of which 27 were cefotaxime-resistant. All 27 cefotaxime-resistant *E. coli* isolates were also uniformly resistant to amoxicillin, cefazoline, cefotaxime, and cefriaxone. High resistance rates were observed for cefuroxime (n=26), moderate for ciprofloxacin (n=8), and low for ceftazidime (n=4) and cefepime (n=1). Only two isolates were resistant to gentamicin. There was no resistance to imipenem and meropenem (Table 2).

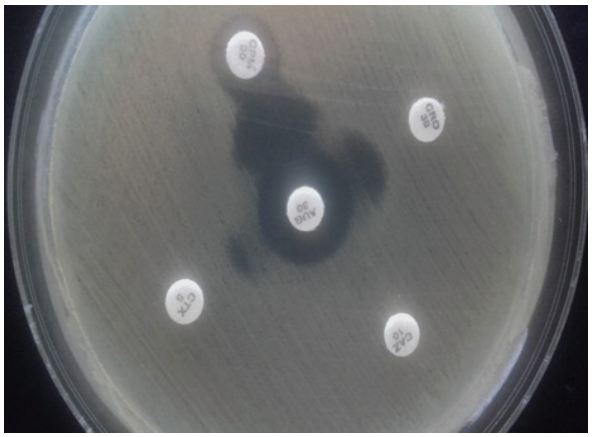

Figure 1 Phenotypic detection of ESBL producers with DDST. The elliptical inhibitory zone between cephalosporins and clavulanic acid (augmentin) indicates an ESBL-producing organism. AUG – augmentin; CAZ – ceftazidime; CPM – cefepime; CRO – ceftriaxone; CTX – cefotaxime

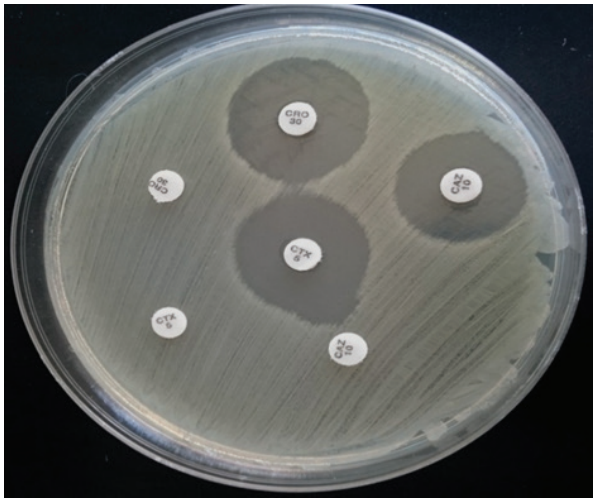

Figure 2 Phenotypic detection of ESBL-producing organisms with the combined disk test. Positive results are those with visible inhibition zones (>5 mm in diameter) around CAZ, CTX, and CRO disks in combination with clavulanate *vs* those without clavulanate. CAZ – ceftazidime; CRO – ceftriaxone; CTX – cefotaxime

Cefoxitin, amikacin, ertapenem, and cotrimoxazole showed good activity with the disk-diffusion method (Table 3).

All cefotaxime-resistant isolates tested positive for ESBLs with both the DDST (Figure 1) and combined disk test (Figure 2), with the enlargement of the inhibition zones around cephalosporins ranging from 5 to 22 mm in the presence of clavulanic acid. The CIM test (Figure 3) confirmed production of ESBL in all tested organisms, with no inhibition zone around cefotaxime disks. Four isolates demonstrated resistance to cefoxitin and were positive in the combined disk test with cloxacillin, with inhibition zones ranging from 10 to 14 mm, which pointed to pAmpC (Figure 4).

Conjugation

Eighteen of the 27 isolates transferred cefotaxime resistance to the *E. coli* recipient strain with the frequency ranging from 5.6×10^{-6} to 5×10^{-4}. Alongside cefotaxime, tetracycline resistance was cotransferred from six, cotrimoxazole from two, and gentamicin resistance from one isolate (Table 4). Ciprofloxacin resistance was not transferred from any of the tested strains.

Molecular detection of resistance genes

PCR identified $bla_{CTX\text{-}M\text{-}1}$ cluster genes in 21 isolates, of which seven tested positive for $bla_{CTX\text{-}M\text{-}15}$. Fourteen isolates tested positive for bla_{TEM} and none for bla_{SHV} genes. IS*Ecp* was found in only one isolate, whereas I*S26* tested negative. The same *bla* genes were found in donor isolates and their respective transconjugants. Six isolates did not harbour any *bla* genes in spite of being phenotypically positive for ESBLs, as shown in Table 2. All but six isolates harboured $bla_{CTX\text{-}ML}$ genes, and 14 harboured additional bla_{TEM} gene.

Table 2 Antibiotic minimum inhibitory concentrations, genes, plasmids, and genotyping of *E. coli* isolates from poultry farms in Bosnia and Herzegovina

No.	Protocol number	Minimum inhibitory concentrations (mg/L) and resistance breakpoint (≥) of antibiotics													Genes, plasmids, and genotypes		
		AMX >32	AMC 128/16	TZP >128/4	CZ >4	CXM >32	CAZ >32	CTX >4	CRO >4	FEP >32	IMI >4	MEM >4	GM >16	CIP >4	ESBL	*bla* genes	PBI and ST
1	31	>128	8	32	>128	>128	64	>128	>128	64	0.5	0.25	>128	>128	+	TEM, CTX-M-15	FIA
2	32	>128	32	8	>128	32	0.5	32	32	0.25	0.12	0.06	2	0.25	+	ND	ND
3	35	>128	1	2	>128	>128	2	>128	>128	0.5	0.06	0.06	0.5	2	+	CTX-M-1 cluster	FIA
4	36	>128	4	8	>128	>128	2	64	64	1	0.06	0.06	1	0.25	+	CTX-M-1 cluster	FIB, ST 117
5	37	>128	8	16	>128	>128	1	>128	64	1	0.12	0.06	1	0.5	+	ND	FIB
6	39	>128	4	8	>128	>128	2	64	64	0.5	0.12	0.12	2	0.12	+	TEM, CTX-M-15	ND
7	40	>128	8	4	>128	>128	0.5	>128	>128	0.5	0.06	0.06	1	0.25	+	ND	ND
8	41	>128	1	4	>128	32	1	64	32	1	0.25	0.12	2	0.25	+	CTX-M-1 cluster	ND
9	43	>128	16	16	>128	>128	8	>128	>128	1	0.25	0.06	4	0.06	+	ND	ND
10	44	>128	32	16	>128	>128	32	>128	>128	2	0.06	0.06	0.5	0.12	+	ND	FIB
11	45	>128	16	16	>128	>128	1	>128	>128	0.5	0.5	0.06	2	0.12	+	CTX-M-1 cluster	ND
12	46	>128	8	4	>128	>128	2	>128	64	1	0.12	0.12	4	0.06	+	TEM, CTX-M-1 cluster	ND
13	47	>128	8	8	>128	>128	16	64	64	1	0.06	0.06	1	0.25	+	TEM, CTX-M-1 cluster	I1
14	49	>128	4	16	>128	>128	4	>128	>128	16	0.25	0.06	0,5	16	+	TEM, CTX-M-1 cluster	I1
15	50	>128	8	16	>128	>128	8	>128	>128	16	0.25	0.12	2	64	+	TEM, CTX-M-15	I1, FIB
16	51	>128	2	4	>128	>128	0.5	>128	>128	0.5	0.12	0.12	2	0.25	+	ND	FIB
17	53	>128	2	4	>128	>128	8	>128	>128	8	0.25	0.06	1	0.25	+	TEM, CTX-M-15	ND
18	54	>128	4	2	>128	>128	1	>128	64	0.5	0.5	0.06	0.5	0.12	+	TEM, CTX-M-15	I1, FIB
19	55	>128	1	2	>128	>128	1	>128	>128	2	0.06	0.06	0.5	0.5	+	TEM, CTX-M-1 cluster	I1
20	56	>128	4	32	>128	>128	16	>128	>128	16	0.5	0.25	2	4	+	TEM, CTX-M-1 cluster	I1, HI1
21	58	>128	2	2	>128	>128	2	>128	>128	8	0.25	0.25	1	0.25	+	CTX-M-1 cluster	ND
22	59	>128	32	4	>128	64	0.5	>128	64	0.5	0.12	0.12	16	32	+	TEM, CTX-M-1 cluster	I1
23	60	>128	32	16	>128	>128	16	>128	32	2	0.5	0.25	1	32	+	CTX-M-1 cluster	I1, HI1, ST155
24	64	>128	32	64	>128	16	>128	>128	32	4	1	0.5	2	>128	+	TEM, CTX-M,-1 cluster	I1, FIB
25	65	>128	4	8	>128	>128	2	64	32	2	0.06	0.06	0.25	0.12	+	TEM, CTX-M-1 cluster	I1
26	66	>128	32	4	>128	32	>128	>128	16	1	0.06	0.06	0.5	64	+	TEM, CTX-M-1 cluster	FIB
27	69	>128	8	2	>128	>128	2	>128	>128	2	0.25	0.12	0.25	0.12	+	CTX-M-15	FIA

AMX – amoxicillin; AMC – amoxicillin/clavulanic; acid; TZP – piperacillin/tazobactam; CZ – cefazolin; CXM – cefuroxime; CAZ – ceftazidime; CTX – cefotaxime; CRO – ceftriaxone; FEP – cefepime; IMI – imipenem; MEM – meropenem; GM – gentamicin; CIP – ciprofloxacin; ESBL – inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases; BL – beta-lactamase content; PBI – plasmid incompatibility group, ST – sequence type; ND – not detected

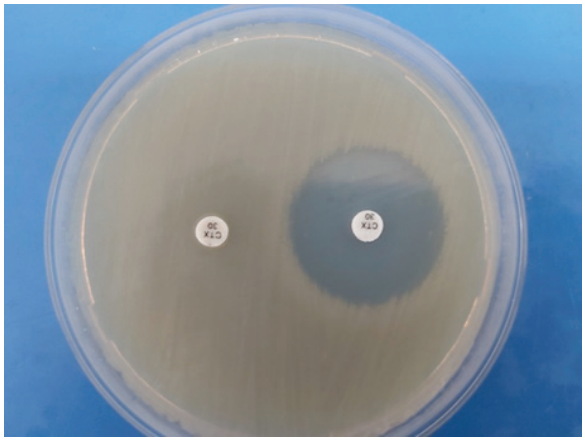

Figure 3 Cephalosporin inactivation method (CIM) with the control *E. coli* ATCC 25922 strain. Left: cefotaxime disk (10 mg) was placed in a heavy suspension of test strains. Right: cefotaxime disk on the surface of the plate as negative control

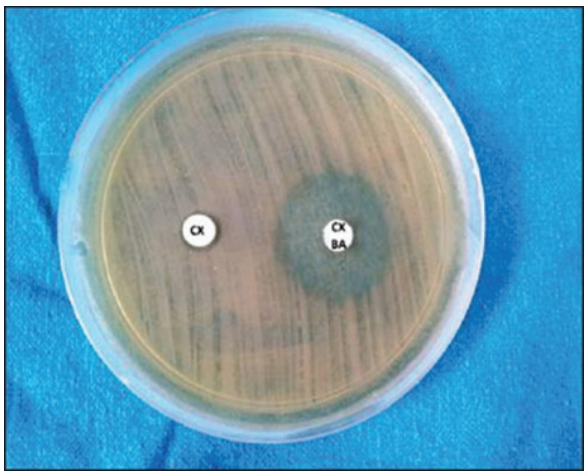

Figure 4 Combined disk test for the detection of plasmid-mediated AmpC β-lactamases with the cefotaxime disk alone (left) and in combination with 3-aminophenylboronic acid (right)

Since bla_{TEM} genes were not sequenced, it was not possible to distinguish between broad spectrum TEM-1 and TEM-2 β-lactamases and their ESBL variants. PCR for *qnr* genes yielded no amplicons.

Plasmid incompatibility groups

The most frequent plasmid incompatibility group was IncFIB, identified in eight isolates, followed by IncFIA (n=3) and Inc HI1 (n=2) (Table 2). Plasmids were not found in nine isolates.

Genotypes

Two different STs were identified: ST117 (*E. coli* 4) and ST155 (*E. coli* 23) (Table 2).

DISCUSSION

Unlike some other studies (11, 12), we found only the CTX-M-1 cluster β-lactamases, which corresponds to those found in *E. coli* isolates identified in humans in this geographic region and highlights the One Health concept of interdependence between human and animal health and the health of their environment. SHV and TEM variants, prevailing in the early 2000s, gave way to the CTX-M family, with *blaCTX-M-15* being the most frequent allelic in clinical isolates of human origin in Bosnia and Herzegovina over the last decade (38). This indicates the possibility that farm animals get colonised with ESBL isolates from farm workers handling the animals, although there are no bibliographical references to support that mode of transmission. Previous studies have demonstrated the opposite direction of transmission, that is, from broilers to farm workers (39, 40).

Whichever the direction, transmission between humans and broilers can occur at any point, even without antibiotic

Table 3 Antibiotic susceptibility of *E. coli* isolates established with the disk-diffusion test

Isolate No.	Protocol number	Disk-diffusion method			
		FOX	AMI	ERT	SXT
1	31	S	S	S	R
2	32	S	S	S	S
3	35	S	S	S	S
4	36	S	S	S	S
5	37	S	S	S	S
6	39	S	S	S	S
7	40	S	S	S	S
8	41	S	S	S	S
9	43	S	S	S	S
10	44	S	S	S	S
11	45	S	S	S	S
12	46	S	S	S	S
13	47	S	S	S	S
14	49	S	S	S	S
15	50	S	S	S	S
16	51	S	S	S	S
17	53	S	S	S	S
18	54	S	S	S	S
19	55	S	S	S	S
20	56	S	S	S	R
21	58	R	S	S	S
22	59	S	S	S	S
23	60	R	S	S	S
24	64	R	S	S	S
25	65	S	S	S	S
26	66	R	S	S	S
27	69	S	S	S	S

AMI – amikacin; ERT – ertapenem; FOX – cefoxitin; R – resistant; S – susceptible; SXT – sulphametoxazole-trimethoprim

pressure, and therefore presents a serious public health issue (41).

Even though the CTX-M-1 cluster was dominant in our poultry isolates, similar to the report in broilers and humans working on the farms in the Netherlands (42, 43) and Hungary (44), the MICs of ceftazidime much lower than those reported in human isolates, and resistance to non-β-lactam antibiotics, usually mediated by the same plasmids coding for ESBLs, was rare.

Six isolates were phenotypically positive for ESBLs, but yielded no product with primers specific for common types of ESBLs, as they produced either rare ESBL types (like VEB, GES, or IBC) not analysed in our study or false positive results.

To our surprise, we found IS*Ecp* in only one isolate, in spite of high bla_{CTX-M} gene transfer and the fact that this insertion sequence promotes CTX-M mobility and expression. However, its lack is in line with low ceftazidime and cefepime MICs.

Four isolates in our study exhibited cefoxitin resistance and tested positive in inhibitor-based test with PBA, which points to the production of pAmpC, but the PCR for common AmpC β-lactamases was negative. This points to the low specificity of phenotypic tests. Similarly, nine isolates exhibited resistance to ciprofloxacin, but the PCR for *qnr* genes yielded no products. A likely explanation is that this resistance was due to chromosomal mutations of the *gyr*A and *par*C genes as previously reported in other Enterobacterales (45). This is in line with the fact that quinolone resistance was not transferable.

Five isolates were resistant to a combination of amoxicillin and clavulanic acid, but we found no inhibitor-resistant TEMs. This resistance may therefore be owed to ESBL overexpression reported in human CTX-M producing *E. coli* isolates (46).

IncI1 plasmid identified in some of the isolates and their transconjugants was previously identified in TEM-52-producing *Salmonella enterica* in Belgian and French poultry (47). This proves the ability of plasmids to acquire various resistance genes and spread them among different Enterobacterales. IncFIA plasmid was found to carry the $bla_{CTX-M-15}$ gene in human *E. coli* isolates from Croatia (48).

The same plasmid showed co-transfer of tetracycline resistance. This warrants for caution in the application of tetracycline in chicken food, as it can exert selection pressure for the transfer of ESBL-encoding plasmids.

In this study, two different STs were found: ST117 and ST155.

CONCLUSION

This study showed that chickens act as a reservoir of ESBL-producing strains and pose a health risk to humans. Interestingly, the isolates possessed the same ESBLs as previously reported in humans from the same region. Although epidemiological links are likely to exist between livestock and farm workers, one limitation of our study is that it did not look for human ESBL-producing *E. coli* to investigate their transfer to/from farm workers. Other limitations include a relatively small number of isolates and genotyping of only two isolates instead of all 27 that would include methods such as pulsed-field gel electrophoresis or rep-PCR. Moreover, we did not sequence the bla_{TEM} *genes* and therefore could not distinguish between the broad-spectrum TEM-1 and TEM-2 β-lactamases and TEM ESBLs.

Even with these limitations, however, our study provides a detailed molecular analysis of resistance determinants from a lot of farms, and therefore an insightful overview of ESBL distribution in Bosnian and Herzegovinian poultry. It also highlights the need for parallel studies of antimicrobial resistance in humans and animals. With that

Table 4 Conjugation frequency and cotransferred resistance markers

Isolate No.	Protocol number	Frequency	Cotransferred resistance markers
1	31	8×10^{-5}	Gm, Smx
2	32	5.5×10^{-4}	
3	35	3.5×10^{-5}	
4	36	1.2×10^{-4}	
5	37	1.4×10^{-5}	
6	39	8.4×10^{-5}	
7	40	4.5×10^{-5}	
8	41	0	
9	43	10^{-4}	Tet
10	44	5×10^{-4}	Tet
11	45	1.6×10^{-6}	Tet
12	46	4.5×10^{-6}	Tet
13	47	1.6×10^{-4}	Tet
14	49	0	
15	50	0	
16	51	7.2×10^{-6}	Smx
17	53	1.57×10^{-5}	
18	54	0	
19	55	5.6×10^{-6}	
20	56	0	
21	58	0	
22	59	0	
23	60	0	
24	64	1.5×10^{-4}	
25	65	1.2×10^{-4}	
26	66	3.1×10^{-5}	Tet
27	69	0	

Gm – gentamicin; Smx – sulphamethoxazole-trimetoprim; Tet – tetracycline

in mind, our future research will involve human subjects working with livestock to compare human and animal isolates and gauge the threat for public health.

Conflicts of interest

None to declare.

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Detekcija i karakterizacija beta laktamaza proširenog spektra i plazmidnih AmpC beta-laktamaza u izolatima *Escherichia coli* s peradarskih farmi u Bosni i Hercegovini

Beta-laktamaze proširenog spektra djelovanja (ESBL) enzimi su koji izazivaju rezistenciju na peniciline, na prvu, drugu, treću i četvrtu generaciju cefalosporina i na aztreonam. Osim u ljudskim uzorcima, ESBL-pozitivni izolati pronađeni su i u hrani životinjskoga podrijetla. Cilj istraživanja bio je analizirati suspektne ESBL-producirajuće izolate dobivene na peradarskim farmama u Zeničko-dobojskom kantonu u Bosni i Hercegovini. Osjetljivost na antibiotike određena je disk-difuzijskom bujonskom mikrodilucijskom metodom. ESBL-producirajući izolati detektirani su metodom dvostrukoga diska i metodom kombiniranih diskova s klavulanskom kiselinom. Prenosivost rezistencije na cefotaksim određena je metodom konjugacije u bujonu. Za detekciju gena koji kodiraju ESBL, plazmidne AmpC beta-laktamaze i determinante rezistencije na kinolone primijenjena je lančana reakcija polimeraze (PCR, od engl. *Polymerase Chain Reaction*). Na 25 peradarskih farmi u Zeničko-dobojskom kantonu ukupno je prikupljeno 108 uzoraka fecesa (obrisci kloake). Od 108 brisova, njih 75 (69,4 %) bilo je pozitivno na *E. coli*, od čega je 27 rezistentno na cefotaksim. Izolati su pokazali otpornost na amoksicilin, cefazolin, cefotaksim i cefriakson te osjetljivost na imipenem, meropenem, ertapenem i amikacin. U dvadeset šest izolata *E. coli* primjenom fenotipskih testova potvrđena je produkcija ESBLs-a. Rezistencije na cefotaksim prenesena je s osamnaest izolata na *E. coli* recipijent soj. PCR-om su utvrđeni bla_{CTX-M} geni, koji pripadaju grupi 1, u dvadeset jednog izolata, od kojih je pet bilo pozitivno na $bla_{CTX-M-15}$. Četrnaest izolata imalo je pozitivan test na bla_{TEM} gene. Najčešća inkompatibilna grupa plazmida bila je IncFIB, a IncFIA i Inc HI1 zastupljeni su u nekoliko izolata. Identificirane su dvije različite klonske skupine, i to ST: ST117 i ST155. Pojava ESBL-producirajućih *E. coli* izolata u domaćih životinja opasnost je za javno zdravlje jer mogu izazvati kolonizaciju crijeva i posljedične infekcije u ljudi.

KLJUČNE RIJEČI: ESBL; javno zdravlje; mikrobna otpornost; ST117; ST155