ESBL and AmpC beta-lactamase-producing *Enterobacteriaceae* in poultry in the Czech Republic

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ABSTRACT: A major reason for resistance of *Enterobacteriaceae* to beta-lactam antibiotics is production of ESBLs and AmpC beta-lactamases. As their more detailed description in poultry is unavailable in the Czech Republic, the presented study aimed at assessing their occurrence and molecular characteristics. A total of 154 composite samples from broilers and 150 cloacal swabs from turkeys were examined. Production of ESBLs was detected in seven *Escherichia coli* isolates and AmpC enzymes in two *E. coli* isolates. The most frequent ESBL types were CTX-M-1 and SHV-12 and the most common AmpC enzymes were the CMY-2 types.

Keywords: *Escherichia coli*; ESBL; AmpC; poultry; Czech Republic

Great attention has been paid to bacterial resistance to antibiotics in both human and animal populations for its adverse impacts on morbidity and mortality from diseases caused by resistant bacteria, economic costs of therapy and real risks of the spread of resistant strains among animals and humans (White, 2006). In gram-negative bacteria, one of the important mechanisms of resistance is the production of beta-lactamases, which inhibit protein transpeptidases participating in bacterial cell wall synthesis (Bradford, 2001; Paterson and Bonomo, 2005). Currently, numerous such enzymes are known and more continue to be described. Of particular clinical and epidemiological importance are ESBLs and AmpC beta lactamases, capable of inactivating the effects of broad-spectrum cephalosporins and penicillins. Production of these enzymes in clinically significant *Enterobacteriaceae* represents an increasing problem resulting in higher patient morbidity and mortality (Paterson and Bonomo, 2005). For example, a recent study described a mortality rate of as high as 60% in patients with bloodstream infections due to ESBL-positive *Enterobacteriaceae* when adequate antibiotic therapy was not administered (Tumbarello et al., 2007).

In humans, *Enterobacteriaceae* which produce ESBLs and AmpC broad-spectrum beta-lactamases have been studied for more than two decades (Bradford, 2001; Paterson and Bonomo, 2005). Similarly, articles describing their prevalence in food animals and foods of animal original have been published in recent years (Brinas et al., 2003; Liebana et al., 2004; Weill et al., 2004; Hasman et al., 2005; Jensen et al., 2006; Wu et al., 2008).

In the Czech Republic, ESBLs and AmpC beta-lactamases were first described by Bardon et al. (2009) in samples from poultry. More detailed data, including the molecular characteristics of these enzymes are unavailable. Therefore, this study aimed at completing this gap.

MATERIAL AND METHODS

Two types of samples were collected for analysis. The first group comprised caecal samples obtained from broilers aged 35 to 40 days at approved poultry slaughterhouses in the Czech Republic. To evaluate seasonal prevalence, slaughterhouse samples (four poultry slaughters, 72 farms) were taken once a month over the entire year 2008. From a slaughter batch (a single farm), 10 caecum samples were collected from birds randomly selected from the batch. After collection, caecal samples in groups of 10 were put in sterile plastic bags and transported to the laboratory. A total of 154 composite samples of broiler caecum contents were evaluated. In the laboratory, the composite samples were mixed and inoculated onto chromID ESBL selective medium (bioMérieux), which can be characterized as chromogenic agar designed for the isolation and detection of ESBL-producing *Enterobacteriaceae*. On chromID ESBL, AmpC-positive *Enterobacteriaceae* can be also detected (Reglier-Poupet et al., 2008)

The other samples were cloacal swabs taken from turkeys immediately after being killed at slaughterhouses in 2008–2009. The turkeys were approximately 12 weeks old, weighed about 10–13 kg and were from three different farms. A total of 150 samples of turkey cloacal swabs were evaluated. The cloacal swabs were collected in three series – in September 2008 (50 swabs), March 2009 (50 swabs) and May 2009 (50 swabs). Immediately after collection, the samples were sent to the laboratory and subsequently inoculated onto the chromID ESBL selective medium (bioMérieux). Minimum inhibitory concentrations (MICs) were determined by the microdilution method (Bardon et al., 2009).

The chromID ESBL selective media were incubated aerobically at 37°C for 24 h. All suspect colonies were isolated onto blood agar (bioMérieux) and then identified using the Phoenix automated system (Becton Dickinson).

Phenotypic detection of ESBL production was performed using a modified double-disk synergy test (mDDST). Jarlier's DDST was modified by including a disk with cefepime and by simultaneous use of a disk with ceftazidime and clavulanic acid (Jarlier et al., 1988; Tzelepi et al., 2000, Pitout et al., 2003).

For detection of AmpC beta-lactamases, a modified AmpC disk test was applied, with ceftazidime, ceftazidime + clavulanic acid and their combination with 400 µg of 3-aminophenylboronic acid. Positive production was detected if inhibition zones were extended by more than five mm when compared with disks without 3-aminophenylboronic acid (Yagi et al., 2005).

Phenotypic testing for ESBL-positive strains was confirmed by PCR detection of genes encoding the appropriate beta-lactamases. Genomic DNA was prepared from an overnight culture (16 h, 37°C) on a meat-peptone agar. Two colonies were resuspended in 100 µl of water and heated to 99°C for 15 min. After removing cellular debris by centrifugation at 13 000 × g for two min, the supernatant was used as template DNA for amplification. PCR analysis of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes was performed using specific primers (Table 1; Arlet et al., 1995; Chanawong et al., 2000; Pagani et al., 2003).

Multiplex PCR was carried out to confirm the production of AmpC beta-lactamases (Table 2; (Perez-Perez and Hanson, 2002). The resulting PCR products were subjected to electrophoretic separation in a 1.5% agarose gel. Visualization of the amplicons was completed by staining with ethidium bromide (Sigma-Aldrich) (1 µg/ml) under UV illumination.

PCR-positive amplicons were purified with the QIAquick PCR Purification Kit (50) (Qiagen) and directly sequenced using amplification primers on the 3130 Genetic Analyzer (Applied Biosystems). Purification and sequencinq was carried out by Genex CZ, s.r.o. Sequence alignment and analysis were performed online using the BLAST program of

Table 1. Oligonucleotide primers used for ESBL detection

Target gene		Primer (nucleotide sequence)
bla_{TEM}	TEM-F	5'-ATGAGTATTCAACATTTCCG-3´
	TEM-R	5´-CCAATGCTTAATCAGTGAGC-3´
bla_{SHV}	SHV-F	5´-CTTTACTCGCTTTATCG-3´
	SHV-R	5´-TCCCGCAGATAAATCACCA-3´
bla_{CTX-M}	CTX-MU1	5´-ATGTGCAGYACCAGTAARGT-3´
	CTX-MU2	5´-TGGGTRAARTARGTSACCAGA-3´

Table 2. Specific set of primers for detection of AmpC beta-lactamases

Target beta-lactamase		Primer (nucleotide sequence)
MOX-1, MOX-2, CMY-1, CMY-8 – CMY-11	MOXMF	5'-GCTGCTCAAGGAGCACAGGAT-3'
	MOXMR	5'-CACATTGACATAGGTGTGGTGC-3'
LAT-1 – LAT-4, CMY-2 – CMY-7, BIL-1	CITMF	5'-TGGCCAGAACTGACAGGCAAA-3'
	CITMR	5'-TTTCTCCTGAACGTGGCTGGC-3
DHA-1, DHA-2	DHAMF	5'-AACTTTCACAGGTGTGCTGGGT-3'
	DHAMR	5'-CCGTACGCATACTGGCTTTGC-3'
ACC	ACCMF	5'-AACAGCCTCAGCAGCCGGTTA-3'
	ACCMR	5'-TTCGCCGCAATCATCCCTAGC-3'
FOX-1–FOX-5b	FOXMF	5'-AACATGGGGTATCAGGGAGATG-3'
	FOXMR	5'-CAAAGCGCGTAACCGGATTGG-3'
MIR-1T, ACT-1	EBCMF	5'-TCGGTAAAGCCGATGTTGCGG-3'
	EBCMR	5'-CTTCCACTGCGGCTGCCAGTT-3'

the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Molecular typing was performed by pulsed-field gel electrophoresis (PFGE) of bacterial DNA. Genomic DNA was isolated according to procedures described by Shi et al. (1996) and digested overnight with *Xba*I. PFGE was run in 1.2% gel for 24 h at 6 V/cm and pulse times of 2–35 s. After PFGE, the gel was stained with ethidium bromide (Sigma-Aldrich) (1 µg/ml) and visualized under UV illumination. The acquired restriction maps of individual isolates were compared using the GelCompar II software.

GelComparII, version 2.0 (Applied Maths, Kortrijk, Belgium) was used to determine the genetic relationship of the strains. DNA bands of each fingerprint pattern were defined manually with a band-position tolerance of 1.5% being the optimal settings needed to define the molecular size marker bands as 100% identical. Similarity coefficients were calculated by using the Dice algorithm, and cluster analyses were performed by the UPGNA algorithms by using the option of the GelComparII program.

RESULTS

In the broiler group, a total of 154 composite samples inoculated onto the chromID ESBL selective medium were studied. In all isolates, the above-mentioned phenotypic tests for the detection of ESBLs and AmpC were performed and three

Table 3. Molecular characterization of ESBL- and AmpC-positive isolates

Isolate No.	Species	Origin	Phenotype	Beta-lactamase(s)
476		broiler	AmpC	CMY-2, TEM-1
483		broiler	AmpC	CMY-2, TEM-1
485		broiler	ESBL	CTX-M-14, TEM-1
K3		turkey	ESBL	SHV-12
K8	*E. coli*	turkey	ESBL	SHV-12
K14		turkey	ESBL	CTX-M-1
K11		turkey	ESBL	CTX-M-1
K27		turkey	ESBL	CTX-M-1
K48		turkey	ESBL	SHV-12

Table 4. MICs of selected antimicrobial agents in ESBL- and AmpC-positive *Escherichia coli* isolates

Isolate No.	Antimicrobial agent/MIC value (mg/l)									
	Origin	TET	MER	GEN	TOB	AMI	OFL	CIP	TIG	COL
476	broiler	4	≤ 0.5	4	2	4	16	8	≤ 0.06	0.5
483	broiler	32	≤ 0.5	2	2	2	16	8	≤ 0.06	0.5
485	broiler	0.5	≤ 0.5	2	2	4	0.25	0.06	≤ 0.06	0.125
K3	turkey	> 32	≤ 0.5	0.5	2	4	16	> 16	0.25	0.5
K8	turkey	32	≤ 0.5	0.5	1	2	16	> 16	0.125	0.5
K14	turkey	2	≤ 0.5	0.5	1	2	≤ 0.125	≤ 0.125	≤ 0.06	1
K11	turkey	2	≤ 0.5	1	2	4	≤ 0.125	≤ 0.125	≤ 0.06	0.5
K27	turkey	2	≤ 0.5	≤ 0.25	0.5	2	≤ 0.125	≤ 0.125	≤ 0.06	0.5
K48	turkey	32	≤ 0.5	0.5	1	2	16	> 16	0.25	0.5

TET = tetracycline, MER = meropenem, GEN = gentamicin, TOB = tobramycin, AMI = amikacin, OFL = ofloxacin, CIP = ciprofloxacin, TIG = tigecycline, COL = colistin

strains producing broad-spectrum beta-lactamases were identified. One strain was ESBL-producing *Escherichia coli*, the other two were *Escherichia coli* which produced AmpC beta-lactamases. From a total of 150 cloacal swabs in turkeys, six ESBL-positive *Escherichia coli* strains were obtained, all of them from a single farm.

Based on PCR assays on *Escherichia coli* isolates with phenotypically confirmed ESBL production, three isolates yielded a positive amplicon with SHV and four with CTX-M primers. Although this method revealed the bla_{TEM} gene in some samples, direct sequencing did not confirm TEM-type ESBLs. The most frequent extended-spectrum beta-lactamases in this group were CTX-M-1 and SHV-12. CTX-M-14 belonging to the CTX-M-9 cluster was detected only in one strain. Multiplex PCR revealed the presence of AmpC enzymes in isolates determined as AmpC producers by phenotypic testing. In both cases, the presence of CMY-2 was indicated. The results of molecular genetic analysis are listed in Table 3.

Table 4 gives the MICs of selected antimicrobial agents. Five strains showed simultaneous resistance to fluoroquinolones and tetracycline. On the other hand, all isolates were susceptible to the tested aminoglycosides, meropenem, tigecycline and colistin.

The results of PFGE are shown in Figure 1. In two groups of tested *Escherichia coli* strains isolated from turkeys in one farm (K8, K48 and K3; K14 and K27), the same restriction profile is evident and these isolates may be considered identical. In broiler isolates, only unique restriction profiles were detected.

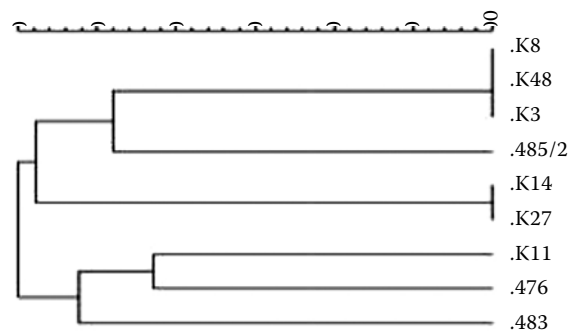Jaccard (Tot 1.5%–1.5%) (H = 0.0%, S = 0.0%) (0.0% –100.0%)
PFGE

Figure 1. Results of pulsed-field gel electrophoresis

DISCUSSION

This study results show that in broilers, two AmpC-positive *Escherichia coli* strains and one ESBL-positive *Escherichia coli* strain were isolated on chromogenic selective agar from 154 composite samples, i.e., from 1540 animals. From cloacal swabs of 150 slaughtered turkeys, six ESBL-positive *Escherichia coli* strains were isolated on chromogenic selective agar, all from a single farm. The presence of ESBL-positive *Enterobacteriaceae* in poultry was also documented by Smet et al. (2008). From 489 cloacal samples collected at five different Belgian broiler farms, they isolated *Escherichia coli* strains and identified 133 ESBL-producing isolates, 127 AmpC-producing isolates and 35 strains producing both ESBL and AmpC enzymes. ESBLs were

dominated by the CTX-M group and CTX-M-1 was most frequent; all AmpC enzymes were found to be CMY-2 (Smet et al., 2008). A study aimed at ESBL- and AmpC-positive *Escherichia coli* in poultry was also reported by Blanc et al. (2006). Among 192 *Enterobacteriaceae* strains, ESBL production was detected in 51 isolates and AmpC production in 13 isolates, all of them *Escherichia coli*. In the case of ESBL enzymes, the CTX-M-9 group was most frequently identified, in particular CTX-M-14. The AmpC enzymes were classified as CMY-2 (Blanc et al., 2006). The comparison of our results with the above-mentioned studies clearly shows that *Escherichia coli* strains producing ESBL and AmpC enzymes are less prevalent. This may be due to, among other factors, the use of antibiotics, since the most frequent antimicrobial agents applied in animals in the Czech Republic are tetracyclines (unpublished data). Based on the molecular analysis, the most dominant types of enzymes were CTX-M-1, SHV-12 and CMY-2, which is in accordance with previously published results (Blanc et al., 2006; Smet et al., 2008). On the other hand, according to results published by Smet et al. (2008), SHV-12 were not detected, and Blanc et al. (2006) described only 5 *Escherichia coli* strains from 51 ESBL-positive isolates with production of SHV-12.

Smet et al. (2008) observed that 48.1% of beta-lactamase-producing *Escherichia coli* isolates were resistant to tetracycline. These findings are in accordance with the results of this study; resistance to tetracycline and fluoroquinolones was demonstrated in five from nine (55.6%) *Escherichia coli* strains producing ESBL or AmpC beta-lactamases.

PFGE detected one group of three and one group of two identical *Escherichia coli* strains isolated from turkeys, suggesting clonal spread of *Enterobacteriaceae* producing ESBLs in a given farm. The *Escherichia coli* isolates from broilers are not identical strains.

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