# Occurrence of bacteria with a dangerous extent of antibiotic resistance in poultry in the Central Region of Moravia

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## Abstract

The study aimed to determine the occurrence of *Enterobacteriaceae* producing broad-spectrum beta-lactamases, vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* strains in poultry in Moravia, Czech Republic, including phenotypic and genotypic analyses of the extent of resistance. Using chromogenic screening media, a total of 240 clinical samples collected from poultry and the poultry farm environment were processed. Phenotypic tests identified 23 isolates of broad-spectrum beta-lactamase-producing *Escherichia coli* and one VRE isolate (*Enterococcus faecium* with VanA resistance). Methicillin-resistant *Staphylococcus aureus* strains were not detected. Among the isolates producing broad-spectrum beta-lactamases, 17 produced extended-spectrum beta-lactamases, most frequently CTX-M; the remaining 6 isolates were CIT-type AmpC enzymes. No carbapenemase-producing strains were detected. Pulsed-field gel electrophoresis showed that 21 *E. coli* strains (91%) were genetically unrelated isolates. Increasing resistance of bacteria to antibiotic agents poses a serious issue for both human and veterinary medicine globally. For humans, a potential source of resistant bacteria may be animals or their products entering the human food chain, for example poultry. The presented study extends existing knowledge about the occurrence of resistant bacteria in poultry in Moravia and describes the phenotype and genotype of their resistance to antibiotics.

*Escherichia coli, broad-spectrum beta-lactamases, vancomycin-resistant enterococci, methicillin-resistant Staphylococcus aureus*

Increasing resistance of bacteria to antibiotic agents poses a serious issue for both human and veterinary medicine, referred to as a global threat. Bacterial resistance to the effects of antibiotics stems from numerous causes combined with each other, making the phenomenon even more dangerous. The most important factors contributing to the development of bacterial resistance are the selection pressure of antibacterials, recombination processes leading to an exchange of the genetic material, and the horizontal spread of genetically identical strains of a particular species (Kolář et al. 2001; Kesselová et al. 2005; Urbánek et al. 2007; Htoutou Sedláková et al. 2014).

The above processes also occur in the animal setting; for humans, a potential source of resistant bacteria may be animals or their products entering the human food chain, for example poultry. The selection pressure of antibiotics may be manifested especially in animals routinely given water or feed containing antimicrobial agents. This category includes poultry. However, there are other routes of transmission of resistant bacteria between animals and humans. The examples are transmission via the food chain and direct or indirect contacts of people closely working with animals such as farmers or veterinarians. Important roles are also played by the environment and aquaculture contaminated with manure which are potential reservoirs of resistance genes (Economou and Gousia

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2015). The transmission of multiresistant strains from animals (animal foods) to humans is said to be mainly associated with Gram-negative bacteria producing broad-spectrum beta-lactamases, vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

The present study aimed to determine the prevalence of *Enterobacteriaceae* producing broad-spectrum beta-lactamases, VRE and MRSA in poultry in the Moravian part of the Czech Republic, including phenotypic and genotypic analyses of the extent of resistance of the isolates to antibiotics and determination of the genetic relationship of isolates.

## Materials and Methods

### Sample collection and processing

Between November 2015 and April 2017, a total of 240 samples obtained from the poultry farm environment and the caeca of broilers in Moravia were examined. Samples were collected on twelve farms (10 samples/farm) and six poultry slaughterhouses (20 samples/slaughterhouse). In both cases, the samples were taken from domestic chickens (*Gallus gallus*). Environmental samples (120 samples) were obtained using sterile non-woven fabric covers put on decontaminated shoes and worn by a worker who walked on the bedding in a poultry shed. Subsequently, the shoe covers were put in a sterile bag and sent to a laboratory for analysis. Caecal contents (120 samples) were collected after slaughter at a processing plant and sent in sterile packaging to the laboratory.

In the laboratory, the shoe covers were put in peptone water (Trios, Czech Republic) which was incubated aerobically in a thermostat for 24 h at 37 °C. Then, the liquid medium was inoculated onto a selective chromogenic agar medium.

Caecal contents were inoculated with a loop directly onto a selective chromogenic agar medium. Both groups of samples were cultured using Oxoid chromogenic screening media, namely Brilliance MRSA Agar, Brilliance VRE Agar, Brilliance ESBL Agar and Brilliance CRE Agar (Thermo Fisher Scientific, UK). Inoculated agars were incubated aerobically for 24 h at 37 °C. For each batch, the quality of chromogenic media was verified with relevant reference strains.

### Species identification of isolates

Suspected isolates with characteristic growth on different types of chromogenic media were isolated in pure culture onto blood agar (Trios, Czech Republic) to identify the species using MALDI - TOF MS (Biotyper Microflex, Bruker Daltonics, Germany).

### Phenotypic tests for determination of antibiotic resistance

The identified isolates were subjected to phenotypic tests to determine their resistance to selected antibiotic agents using a standard microdilution method (EUCAST 2016). *Enterobacteriaceae* isolates were tested for resistance to gentamicin, sulphamethoxazole/trimethoprim, colistin, meropenem, ciprofloxacin, tigecycline, tobramycin and amikacin. An *Enterococcus* isolate was tested for resistance to ampicillin, ciprofloxacin, nitrofurantoin, vancomycin, teicoplanin, linezolid, and tigecycline. Reference strains *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used for protocol quality control. For phenotypic detection of broad-spectrum beta-lactamases, a modified double-disk synergy test and AmpC test were used (Htoutou Sedlakova et al. 2011).

### Molecular biology tests for determination of antibiotic resistance

More detailed determination of resistance mechanisms was performed with molecular biology tests detecting and analysing resistance genes. The polymerase chain reaction (PCR) mixture contained 1 µl of deoxyribonucleic acid (DNA) - 100 ng in 24 µl of complete reaction buffer with $MgCl_2$ (containing 100 mmol/l Tris-HCl [pH 8.8], 500 mmol/l KCl, 1% Triton X-100, 15 mmol/l $MgCl_2$) (Top-Bio, Czech Republic; 2.5 µl), deoxyribonucleotide triphosphate (dNTP) - 10 mmol/l, 0.2 µl, 20 pmol of each primer (0.2 µl) and Taq DNA polymerase (Top-Bio; 0.2 µl). The PCR conditions were as follows: initial denaturation at 95 °C for 7 min, 35 cycles at 95 °C for 40 s, at different annealing temperatures for 40 s (Table 1) and 72 °C for 1 min, followed by a single final elongation step at 72 °C for 7 min. The primer sequences, concentrations, and calculated lengths of the corresponding amplicons are listed in Table 1.

### Detection of genetic relatedness of selected isolates

To determine the genetic relatedness of *E. coli* isolates, macrorestriction analysis of chromosomal DNA was performed using pulsed-field gel electrophoresis (PFGE). Genomic DNAs prepared in accordance with a previously described procedure (Husickova et al. 2012) were digested with XbaI (30 U for 3 h) (Takara,

Bio, Otsu, Shiga, Japan) and subjected to PFGE as described elsewhere (Roderova et al. 2016). The resulting restriction profiles of isolates were compared using the GelCompareII software (Applied Maths, Kortrijk, Belgium). A dendrogram was created to determine clonal relatedness among the studied *E. coli* strains.

Table 1. Sequences of primers used for polymerase chain reaction for detection of resistance genes.

| Targeted gene | Primer name | Sequence (5' to 3' direction)[a,b] | Length (bases) | Amplicon size | Tm (⁰C) | Reference |
|---|---|---|---|---|---|---|
| | | *Escherichia coli* | | | | |
| $bla_{TEM}$ type | TEM-F | GCGGAACCCCTATTTG | 16 | 964 bp | 56 | Olesen |
| | TEM-R | ACCAATGCTTAATCAGTGAG | 20 | | | et al. 2004 |
| $bla_{SHV}$ type | SHV-F | CTTTACTCGCCTTTATCG | 18 | 827 bp | 56 | Chanawong |
| | SHV-R | TCCCGCAGATAAATCACCA | 19 | | | et al. 2000 |
| $bla_{CTX-M}$ type | CTX-M-F | ATGTGCAGYACCAGTAARGT | 20 | 593 bp | 56 | Pagani |
| | CTX-M-R | TGGGTRAARTARGTSACCAGA | 21 | | | et al. 2003 |
| $bla_{OXA}$ type | OXA-1F | ACACAATACATATCAACTTCGC | 22 | 813 bp | 56 | Steward |
| | OXA-1R | AGTGTGTTTAGAATGGTGATC | 21 | | | et al. 2001 |
| | OXA-2F | TTCAAGCCAAAGGCACGATAG | 21 | 702 bp | 58 | Steward |
| | OXA-2R | TCCGAGTTGACTGCCGGGTTG | 21 | | | et al. 2001 |
| | OXA-10F | CGTGCTTTGTAAAAGTAGCAG | 21 | 651 bp | 56 | Steward |
| | OXA-10R | CATGATTTTGGTGGGAATGG | 20 | | | et al. 2001 |
| $bla_{AmpC-CIT}$ ($bla_{LAT}$ type, $bla_{CMY}$ type, $bla_{BIL}$ type) | CIT-F | TGGCCAGAACTGACAGGCAAA | 21 | 462 bp | 64 | Perez-Perez and Hanson 2002 |
| | CIT-R | TTTCTCCTGAACGTGGCTGGC | 21 | | | |
| $bla_{AmpC-MOX}$ ($bla_{MOX}$ type, $bla_{CMY}$ type) | MOX-F | GCTGCTCAAGGAGCACAGGAT | 21 | 520 bp | 64 | Perez-Perez and Hanson 2002 |
| | MOX-R | CACATTGACATAGGTGTGC | 19 | | | |
| $bla_{DHA}$ type | DHA-F | AACTTTCACAGGTGTGCTGGGT | 22 | 405 bp | 64 | Perez-Perez and Hanson 2002 |
| | DHA-R | CCGTACGCATACTGGCTTTGC | 21 | | | |
| $bla_{ACC}$ type | ACC-F | AACAGCCTCAGCAGCCGGTTA | 21 | 346 bp | 64 | Perez-Perez and Hanson 2002 |
| | ACC-R | TTCGCCGCAATCATCCCTAGC | 21 | | | |
| $bla_{AmpC-EBC}$ ($bla_{MIR}$ type, $bla_{ACT}$ type) | EBC-F | TCGGTAAAGCCGATGTTGCGG | 21 | 302 bp | 64 | Perez-Perez and Hanson 2002 |
| | EBC-R | CTTCCACTGCGGCTGCCAGTT | 21 | | | |
| $bla_{FOX}$ type | FOX-F | AACATGGGGTATCAGGGAGAT | 21 | 190 bp | 64 | Perez-Perez and Hanson 2002 |
| | FOX-R | CAAAGCGCGTAACCGGATTGG | 21 | | | |
| | | *Enterococcus faecium* | | | | |
| $bla_{VanA/B}$ type | VanA-F | GGGAAAACGACAATTGC | 17 | 732 b | 62 | Dutkamalen et al. 1995 |
| | VanA-R | GTACAATGCGGCCGTTA | 17 | | | |
| | VanB-F | ATGGGAAGCCGATAGTC | 17 | 635 bp | 62 | Dutkamalen et al. 1995 |
| | VanB-R | GATTTCGTTCCTCGACC | 17 | | | |
| | | *Staphylococcus aureus* | | | | |
| $bla_{MecA}$ type | MecA-F | TCCAGATTACAACTTCACCAGG | 22 | 162 bp | 53 | Oliveira and de Lencastre 2002 |
| | MecA-R | CCACTTCATATCTTGTAACG | 20 | | | |

[a]For degenerate primers: R = A or G; S = G or C; Y = C or T. [b]Concentration of each of the primers was 20 pmol.

## Results

A total of 240 samples were processed using various screening media. Among those, phenotypic tests identified 23 isolates (9.6%) producing broad-spectrum beta-lactamases. Additionally, one case of VRE was identified. No MRSA strains were found.

All isolates producing broad-spectrum beta-lactamases were *E. coli.* In 17 isolates, the exended spectrum beta-lactamases (ESBL) phenotype was detected. The CTX-M-type ESBL production was confirmed in 14 strains, with two and four strains being also of the SHV and TEM types, respectively. Additionally, two strains produced both TEM and SHV ESBLs and one strain produced only SHV-type ESBL. The remaining 6 isolates were the CIT-type AmpC enzymes, with the CIT complex comprising, for example, CMY-2/-3/-4, LAT-1/-2, and BIL-1. No carbapenemase-producing strains were found. As seen from Table 2, tests assessing the resistance of *E. coli* isolates to selected antibiotics showed higher resistance to ciprofloxacin and sulphamethoxazole/trimethoprim. No other significant resistance phenotypes were noted. Detailed specifications and prevalence rates for resistance genotypes of *E. coli* strains producing broad-spectrum beta-lactamases are shown in Table 3.

Table 2. Results of resistance tests for *Escherichia coli* (n = 23).

| Tested antibiotic | GEN | COT | COL | MER | CIP | TIG | TOB | AMI |
|---|---|---|---|---|---|---|---|---|
| Susceptible strains (n) | 23 | 16 | 23 | 23 | 9 | 23 | 23 | 23 |
| Resistant strains (n) | 0 | 7 | 0 | 0 | 14 | 0 | 0 | 0 |
| Resistance (%) | 0 | 30.4 | 0 | 0 | 60.9 | 0 | 0 | 0 |

Legend: n – number of tested isolates, GEN – gentamicin, COT – sulphamethoxazole + trimethoprim, COL – colistin, MER – meropenem, CIP – ciprofloxacin, TIG – tigecycline, TOB – tobramycin, AMI – amikacin

Table 3. Prevalence of resistant strains of *Escherichia coli* (n = 23*)* by type of resistance.

| Identified genotype | n | % |
|---|---|---|
| ESBL ($bla_{CTX-M}$) | 8 | 34.8 |
| ESBL ($bla_{SHV}$, $bla_{CTX-M}$) | 2 | 8.3 |
| ESBL ($bla_{TEM}$, $bla_{SHV}$) | 2 | 8.3 |
| ESBL ($bla_{TEM}$, $bla_{CTX-M}$) | 4 | 16.7 |
| ESBL ($bla_{SHV}$) | 1 | 4.2 |
| AmpC (CIT-type) | 6 | 25.0 |

ESBL - extended-spectrum beta-lactamases; n – number of tested isolates

Pulsed-field gel electrophoresis was used to determine the clonality of all 23 tested *E. coli* isolates producing broad-spectrum beta-lactamases (ESBL and AmpC). Twenty-one strains were confirmed to be genetically unrelated isolates. The remaining two CTX-M-type ESBL producers (strains S5113 and S5115) were found to be identical strains based on comparison of their restriction profiles (Fig. 1). Subsequently, the two strains were found to come from different sheds located at the same farm.

Among the 240 tested samples, only one case of VRE was identified. This was an *Enterococcus faecium* isolate with the VanA type of resistance. This strain was only susceptible to linezolid and tigecycline, and resistant to the other tested antibiotics (i.e. ampicillin, ciprofloxacin, nitrofurantoin, vancomycin and teicoplanin).

## Discussion

The occurrence of multiresistant strains in the food chain may lead to colonization of the human intestinal tract, allowing subsequent interhuman transmission. The sources may be animal meat, milk and eggs. In case of primary contamination, the animal itself is the carrier of a multiresistant strain. However, secondary contamination of the food chain
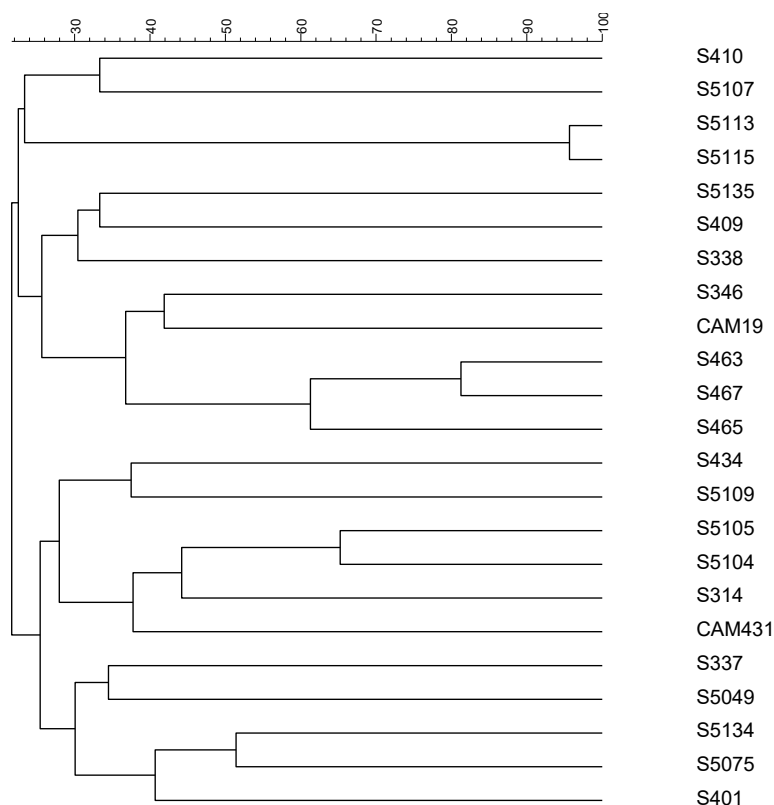
Fig. 1. Dendrogram of broad-spectrum beta-lactamase-producing *Escherichia coli*

may occur if raw materials or foods of animal origin are processed, transported or stored. Special attention should be paid to foods of animal origin which have not been heat-treated. Bacteria with a dangerous degree of resistance to antibiotics that threaten the treatment of human infections are mainly bacteria producing broad-spectrum beta-lactamases (e.g. ESBL, AmpC and carbapenemases in case of *Enterobacteriaceae*), MRSA, VRE and bacteria with resistance to fluoroquinolones (e.g. *E. coli*). Potential carriers of this resistance are bacteria commonly occurring in foods for which no large-scale monitoring has been performed as yet (EFSA 2016). An increased incidence of broad-spectrum beta-lactamase-producing *E. coli* has been observed in food-producing animals such as cattle, chickens and pigs. These findings suggest that animals and foods may be sources of resistant bacteria for humans as well (WHO 2011; R e i c h et al. 2013).

In the present study, broad-spectrum beta-lactamase-producing *E. coli* was isolated in only 9.6% of 240 tested samples (cloacal swabs, environment). For example, E g e a et al. (2012) reported a contamination of raw poultry meat with ESBL-producing *E. coli* of 93.3%. O v e r d e v e s t et al. (2011) found a high prevalence of bacteria carrying genes encoding ESBL in retail chicken meat samples (79.8% of positive samples), with genetic analysis showing that ESBL-positive strains identical with those from human rectal swabs prevailed. The higher prevalence rates of bacteria in poultry meat compared to clinical samples (cloacal swabs) may be due to secondary contamination of meat during slaughter and subsequent manipulation. A similar situation has been observed, for example, in

*Campylobacter* spp., with the prevalence of these bacteria being lower in the caeca than on the animals' injured skin or in fresh poultry meat in retail (Bardoň et al. 2011).

*Escherichia coli* isolates mainly harbour different ESBL genes ($bla_{CTX}$, $bla_{TEM}$ and $bla_{SHV}$) and AmpC genes ($bla_{ACC}$ and $bla_{CMY}$) (Dierikx et al. 2010; Kolar et al. 2010). Furthermore, Randall et al. (2011) showed that many of the chicken or turkey isolates tested were positive for the $bla_{OXA}$ genes (Randall et al. 2011). Moreover, several MCR-1-producing *E. coli* isolates have been detected from chicken meat coproducing either an ESBL (SHV-12) or an AmpC (CMY-2) cephalosporinase, or both (Hasman et al. 2015). In addition, $bla_{DHA}$ and $bla_{ACT-like}$ AmpC beta-lactamases have been reported in *Salmonella enterica* isolated from chicken farms in South Korea and *Klebsiella pneumoniae* in chickens in China, respectively (Rayamajhi et al. 2010; Wu et al. 2012). The presence of ESBL/AmpC genes in *E. coli* from food-producing animals may present a risk to human health. Interestingly, Laube et al. (2013) reported that faeces, litter, and even dust may act as transmission sources of ESBL/AmpC-producing *E. coli* in a broiler barn. Besides, isolation of ESBL-producing *E. coli* from flies has been described, suggesting a potential alternative route of transmission of ESBL-producing *E. coli* from poultry to humans (Blaak et al. 2014). Another factor that could influence the high detection rates of beta-lactamases in the investigated farms is the intensive use of antibiotics. In the present study, the most common genotype identified in *E. coli* isolates was the ESBL $bla_{CTX-M}$ (34.8%), followed by CIT-type AmpC (25%). No strains producing carbapenemases and resistant to colistin were detected.

Analysis of 23 broad-spectrum beta-lactamase-producing *E. coli* isolates failed to show marked clonal spread of this type of resistance on poultry farms. Twenty-one strains were confirmed to be genetically unrelated isolates. Only two CTX-M-type ESBL producers were found to be identical strains. This is consistent with a study by Casella et al. (2017) showing that positive ESBL-producing *E. coli* isolates mostly did not spread clonally.

In the present study, the prevalence of VRE was very low. Among 240 tested samples, only one case of VRE was identified, namely *E. faecium*, subsequently found to have the VanA type of resistance. Our previous research on VRE in poultry in the Olomouc Region, Moravia, showed a higher prevalence of VRE in poultry. In 2002–2004, for example, a total of 527 strains of *Enterococcus* spp. were isolated from 1,050 cloacal swabs collected from poultry in the region. Vancomycin-resistant enterococci were detected in 11 cases (2.1%). Most isolates (54.5%) were identified as *E. faecium* with the VanA type of resistance (Kolář et al. 2005). Compared to the 2002–2004 findings, the prevalence of VRE on poultry farms in the present study is not alarming; however, the monitoring of resistance needs to be continued.

The increasing resistance of bacteria to the effects of antibiotic therapy is a major problem in both veterinary and human medicine. Its solution requires understanding the development and spread of resistance between humans and animals. The essential prerequisite is close interdisciplinary cooperation and surveillance of bacterial resistance; its integral part is the determination of the type of selection, ways and mechanisms of spread of multiresistant bacteria, including the genetic basis.

The presented study extends existing knowledge about the occurrence of resistant bacteria in poultry in Moravia and describes the phenotype and genotype of their resistance to antibiotics. More detailed determination of resistance mechanisms was performed with molecular biology tests especially in the case of broad-spectrum beta-lactamase-producing *E. coli* isolates.

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