Occurrence of antibiotic-resistant bacterial strains isolated in poultry

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ABSTRACT: The main goal of the study was to analyse the occurrence of antibiotic-resistant bacterial strains in poultry in the Czech Republic in 1999–2000. The resistance was determined in 128 selected *Escherichia coli*, 88 *Staphylococcus* sp. and 228 *Enterococcus* sp. strains. The bacterial species were selected to represent gramnegatives and grampositives, the common part of intestinal microflora and also opportunistic pathogens. In *Escherichia coli*, 97% of strains were found to be resistant to tetracycline, 51% were resistant to ampicillin, and 31% were resistant to piperacillin. Increased frequencies of resistance to ofloxacin and ciprofloxacin (in 10% of the strains) were also found. In staphylococci, increased numbers of strains resistant to erythromycin (39%), clindamycin (19%), tetracycline (14%) and ofloxacin (13%) were observed. In enterococci, 80%, 59% and 34% of the strains were resistant to tetracycline, erythromycin or nitrofurantoin, respectively. A high-level resistance to streptomycin was proved in 22% of the strains. Eleven *Enterococcus* sp. strains were found to be resistant to vancomycin (vancomycin-resistant enterococci – VRE). Being of the clinical importance, the VRE strains were analysed in detail. Six VRE were identified as *Enterococcus faecium* VanA, three strains belonged to *Enterococcus* sp. group III VanB. The remaining two strains were classified as *Enterococcus faecium* VanB and *Enterococcus faecalis* VanB, respectively. Based on *Sma*I macrorestriction analysis, regardless of their resistance type, vancomycin-resistant *Enterococcus faecium* strains formed a cluster distinct from the control group of vancomycin sensitive strains. Furthermore, within the cluster of vancomycin resistant *Enterococcus faecium* strains, two clonal lines could be distinguished while the sensitive strains were more heterogeneous.

Keywords: poultry; antibiotics; resistance; vancomycin-resistant enterococcus

The development of resistance to antibiotics in bacteria led to a discussion about the careful use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture (Caprioli *et al.*, 2000). It is now generally known that the main risk factor for an increase in bacterial resistance is an increased use of antibiotics. It is similar in humans and in animals. It is to be stressed that in animals the antimicrobial agents are not used only for therapy and prevention of bacterial infections but also as growth promoters (Bogaard *et al.*, 1997; Bogaard and Stobberingh, 2000). In Europe, approximately 30% of all antibiotics used in animals are growth promoters (Bogaard and Stobberingh, 2000). One of the common conclusions of the EU Conference on the Microbial Threat in 1998 and the Scientific Steering Committee on Antimicrobial Resis-tance in 1999 was a presumption that the use of antimicrobial drugs and the development of resistance in humans and animals are interrelated (Rosdahl and Pedersen, 1998; European Commission, DG XXIV, 1999). It is very important to monitor the resistance to antibiotics not only in human bacterial pathogens but also in pathogenic and commensal bacteria of animal origin.

Among others, the surveillance should include staphylococci, enterococci and enterobacteria (Caprioli *et al.*, 2000). Special attention should be paid to vancomycin-resistant enterococci (VRE) that nowadays

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represent a high-level risk. In the Czech Republic, these strains were recorded in humans as well as in animals (Bergerová and Turková, 1997; Kolář *et al.*, 1997, 2000). In the last decade, enterococci became the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia in humans (Scheberg *et al.*, 1991). For the treatment of serious enterococcal infections in humans, ampicillin and aminoglycosides have been considered as the drugs of choice. Diseases caused by strains that are resistant to ampicillin and aminoglycosides can be treated with glycopeptides. For this reason, the VRE occurrence represents a serious problem.

The source of VRE in humans is not exactly known. One possibility is that these strains are spread via the food chain. In Europe, a diversity of VRE types have been isolated from sewage, animal waste, meat and meat products, suggesting a heterogeneous pool of VRE outside hospitals (Klare *et al.*, 1995; Bates, 1997). The observation that in the United States VRE have not been isolated from animal sources might be due to the fact that glycopeptides have never been approved there for use in animal food as a growth promoter (McDonald *et al.*, 1997). In countries where avoparcin (a vancomycin analogue) was used as a growth promoter, VRE were found not only in animals fed with avoparcin but also in the faecal flora of healthy humans and pet animals (Bogaard *et al.*, 1996, 1997; Van Belkom *et al.*, 1996). It can be supposed that the transmission of VRE to persons who got in contact with these sources results in an increase in the human reservoir of these strains (Bates *et al.*, 1993, 1994; Bogaard *et al.,* 1996; Kruse and Rorvik, 1996).

At present, five types of resistance (A–E) of enterococci to vancomycin were identified (Murray, 1998). The VanA phenotype with high-level inducible resistance to vancomycin and teicoplanin (a result of the acquisition of the *vanA* resistance gene cluster on Tn*1546* or related elements), the VanB phenotype with a lower level of inducible resistance to vancomycin and susceptibility to teicoplanin, and the VanC phenotype due to the chromosomal *vanC* resistance gene in certain species (*Enterococcus gallinarum*, *Enterococcus casseliflavus*). The phenotype VanD was identified in the strain *Enterococcus faecium* with constitutive resistance to vancomycin and susceptibility to teicoplanin (Perichon *et al.*, 1997). The VanE phenotype properties are similar to those of the VanC phenotype (Fines *et al.*, 1999).

The main goal of this study was to analyze the occurrence of antimicrobial resistance in bacterial strains isolated from poultry in the central part of Moravia (Czech Republic). Special attention was paid to vancomycin-resistant enterococci where an analysis of plasmid and genomic DNA was performed and their similarity degree was determined.

MATERIAL AND METHODS

In the years 1999–2000, 1 667 samples of section material (small intestine) and 2 955 samples of clinical material (cloacal smears, faeces) from poultry were examined using aerobic cultivation. The examination was aimed at *Escherichia coli* strains, staphylococci and enterococci. Poultry was bred in the region of central Moravia. Besides, two targeted screenings were performed on the poultry farm P., each of them evaluating 100 samples of cloacal smear.

Each sample was cultivated on conventional nonselective media (blood agar) and selective media (Endo agar) under aerobic conditions. Fermentative gram-negative rods of the family *Enterobacteriaceae* were determined by standard biochemical procedures using Enterotest 24 and Enterotest 16 (Lachema, Czech Republic). Staphylococci were identified according to their growth characteristics on blood agar (Oxoid) and by standard biochemical procedures using Staphytest (Lachema). Enterococci were identified according to the criteria of Facklam and Collins (1989) and by their biochemical activities using En-coccus test (Lachema). All enterococcal strains were positive for the group D antigen (Oxoid).

All isolated enterococci (228 strains) and staphylococci (88 strains) were tested for susceptibility to antibiotics. For *Escherichia coli*, susceptibility was determined in 128 selected strains. Detection of susceptibility to tested antibiotics was performed by the microdilution method in accordance with the NCCLS guideline (National Committee for Clinical Laboratory Standards, 1997). The values of minimum inhibition concentration of vancomycin and teicoplanin were used to determine the phenotype of resistance to vancomycin in enterococci. The reference strains *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as control of quality. Resistance of enterococci to high concentrations of aminoglycosides (HLR) was established using a disk agar method with gentamicin (Sanofi Pasteur, 500 μg) and streptomycin (Sanofi Pasteur, 500 μg) disks.

Isolation of plasmid DNA was performed using High Pure Plasmid Isolation Kit (Roche Diagnostics) according to the supplier's recommendation with minor

modification concerning prolonged lysis for 1 hour and addition of lysozyme (Sigma) to final concentration of 0.5 mg/ml. Plasmid DNA was subjected to slab gel electrophoresis on a 0.8% agarose horizontal gel in 1 × TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.8) at 1.5 V/cm for 6 h at room temperature. Supercoiled DNA ladder (Sigma) was used for the determination of the size of superhelical form of plasmids.

DNA isolation for pulsed-field gel electrophoresis (PFGE) was performed according to the methods of Murray *et al.* (1990) modified by Pantůček *et al.* (1996) in all VRE isolated. Three strains of *Enterococcus faecium* and one strain of *Enterococcus faecalis*, isolated from the farm P. (the source of all VRE) and susceptible to vancomycin, served as a control group. Restriction enzyme cleavage was performed with 8 units of *Sma*I (Roche Diagnostics) (for 1 × 1 × 5 mm agarose blocks) for 12 hours at 30°C. PFGE was performed with the CHEF-MAPPER system (Bio-Rad) in 1.2% (w/v) agarose gels (Qualex Gold Agarose, Angewandte Gentechnologie Systeme) at 14°C in a 1 × TAE buffer. A constant voltage 6 V/cm was applied with increasing pulse time of 5–35 s over a period of 30 hours. Concatemers of bacteriophage λcI857Sam7 (Bio-Rad) were used as size markers. Digitised gel images were analysed using GelCompar 4.1 software (Applied Maths BVBA). Macrorestriction patterns of DNAs isolated from individual strains were compared qualitatively. The UPGMA algorithm showed the best co-phenetic correlations (95–98%) and was therefore selected for constructing the dendrograms from the data of PFGE.

Table 1. Resistance of 128 *Escherichia coli* strains to antibiotics

Antibiotic	Resistance (%)
Amikacin	6
Ampicillin	51
Ampicillin/sulbactam	0
Aztreonam	6
Cefazolin	6
Cefpirome	6
Cefoperazone	6
Cefoperazone/sulbactam	6
Cefotaxime	6
Ceftazidime	6
Cefuroxime	6
Cefoxitin	6
Ciprofloxacin	10
Chloramphenicol	8
Gentamicin	6
Meropenem	6
Netilmicin	6
Ofloxacin	10
Piperacillin	31
Piperacillin/tazobactam	0
Tetracycline	97
Tobramycin	6
Trimethoprim/sulfamethoxazole	14

RESULTS

The total number of 1 794 bacterial strains were isolated from poultry flocks in the central Moravian region during this study. Gram-negative bacteria accounted for 67.6% (1 213 strains) and Gram-positive ones for 32.4% (581 strains). Major species were *Escherichia coli* (61.3%), *Streptococcus* sp. (14.8%), *Enterococcus* sp. (12.7%) and *Staphylococcus* sp. (4.9%). The other isolated microbes (*Salmonella* sp., *Proteus mirabilis, Pasteurella multocida* and *Pseudomonas aeruginosa*) accounted for 6.3%.

Resistance of selected *Escherichia coli* strains (128 strains) to antimicrobial agents is given in Table 1. Resistance to tetracycline reached 97%, to ampicillin 51% and to piperacillin 31%. Higher frequency of resistant strains (10%) was also proved in the case of ofloxacin and ciprofloxacin.

Table 2. Resistance of 88 *Staphylococcus* sp. and 228 *Enterococcus* sp. strains to antibiotics

Antibiotic	Resistance (%)	
	Staphylococcus sp.	*Enterococcus* sp.
Ampicillin	–	3
Ampicillin/sulbactam	4	3
Clindamycin	19	–
Chloramphenicol	3	7
Erythromycin	39	59
Gentamicin (high–level resistance)	–	7
Nitrofurantoin	–	34
Ofloxacin	13	51
Oxacillin	4	–
Streptomycin (high–level resistance)	–	22
Teicoplanin	0	5
Tetracycline	14	80
Vancomycin	0	5

Table 2 shows resistance to antibiotics in *Staphylococcus* (88 strains) and *Enterococcus* (228 strains) species. In staphylococci, increased resistance levels were found for erythromycin (39%), clindamycin (19%), tetracycline (14%) and ofloxacin (13%). In enterococci, higher frequencies of tetracycline-resistant strains (80%), erythromycin-resistant strains (59%) and nitrofurantoin-resistant strains (34%) were found. A high-level resistance to streptomycin was proved in 22% of strains.

In the whole, 11 VRE were evidenced. These strains were isolated only from farm P. at different times during the study. Six strains of them were identified as *Enterococcus faecium* VanA. Three strains were identified as *Enterococcus* sp. group III VanB, one strain was determined as *Enterococcus faecium* VanB and another strain as *Enterococcus faecalis* VanB.

The results of plasmid content analysis of vancomycin-resistant enterococci of animal origin are given in Table 3. *Enterococcus faecium* VanA 1-3 strains contained a large plasmid, the size of which could not be determined by standard gel electrophoresis. *Enterococcus faecium* VanA 4–6 strains were of identical plasmid profile. No plasmids were detected in *Enterococcus faecalis* VanB and in the control group of enterococci susceptible to vancomycin, in *Enterococcus faecium* strains 1–3 and *Enterococcus faecalis* 4.

In VanA vancomycin-resistant strains, 5.2 kbp plasmid (absent in VanB strains) was recorded most frequently. On the contrary, 4.3 kbp plasmid was always present in VanB strains group III (absent in VanA strains).

Figure 1 shows a dendrogram giving the degree of similarity of *Sma*I macrorestriction patterns of the

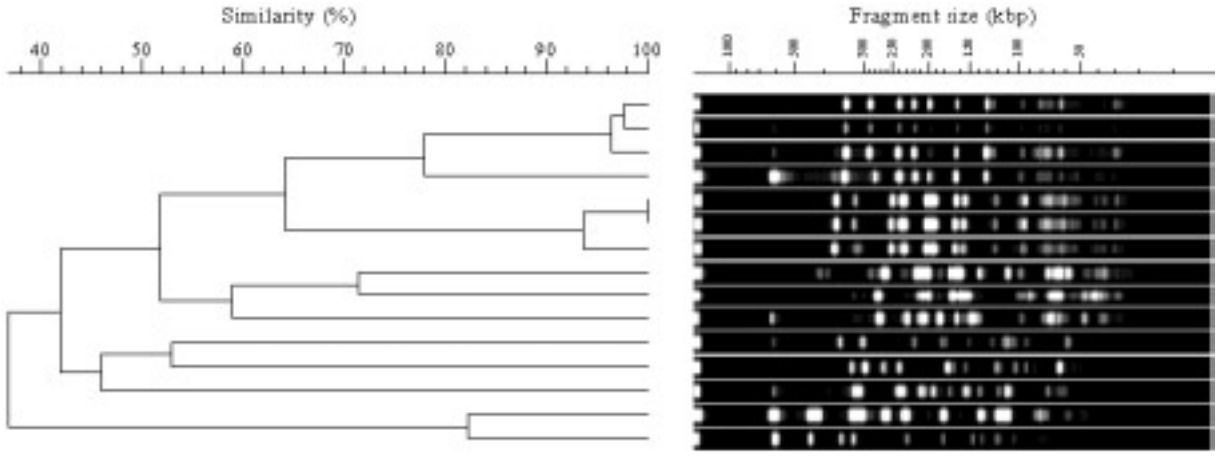

Figure 1. A dendrogram showing the degree of similarity of *Sma*I macrorestriction patterns of the genomic DNAs

The dendrogram contains the following strains arranged from the top downwards:
E. faecium VanA 4, *E. faecium* VanA 5, *E. faecium* VanA 6
E. faecium VanB
E. faecium VanA 1, *E. faecium* VanA 2, *E. faecium* VanA 3

E. faecium susc. 1
E. faecium susc. 2
E. faecium susc. 3

Enterococcus sp. group III VanB 1
Enterococcus sp. group III VanB 2
Enterococcus sp. group III VanB 3

E. faecalis VanB

E. faecalis susc. 4.

Three strains of *Enterococcus faecium* and one strain of *Enterococcus faecalis*, isolated from the farm P. and susceptible to vancomycin (susc.), served as control group

Table 3. Analysis of plasmid content in 11 vancomycin-resistant enterococci

Strain	Approximative plasmid size in kbp										
	>40	21	19	6.2	6.0	5.2	4.3	4.1	3.2	3.0	1.6
E. faecium VanA 1	×			×		×					
E. faecium VanA 2	×				×						
E. faecium VanA 3	×		×			×					
E. faecium VanA 4						×		×			
E. faecium VanA 5						×		×			
E. faecium VanA 6						×		×			
*E. faecium*VanB					×			×			
E. sp. group III VanB 1		×					×				
E. sp. group III VanB 2			×				×				
E. sp. group III VanB 3							×		×	×	×
E. faecalis VanB											

Legend: Presence of plasmid is marked with ×

genomic DNA. Based on the results of the analysis, the strains were divided into five clusters:

1. *Enterococcus faecium* VanA 4–6 and strain *Enterococcus faecium* VanB
2. *Enterococcus faecium* VanA 1–3
3. *Enterococcus faecium* 1–3 susceptible to vancomycin
4. *Enterococcus* sp. group III (B1– B3). Highly heterogeneous group involving non-related strains
5. *Enterococcus faecalis* VanB and strain *Enterococcus faecalis* 4 susceptible to vancomycin

DISCUSSION

The increase in bacterial resistance to antibiotics and rising frequency of bacterial strains with dangerous level of bacterial resistance represent a serious problem nowadays. Applications of antibiotics bring about an increase in resistance to antibiotics not only in pathogenic bacterial strains but also in strains forming a part of the endogenous flora of humans and animals. Multiresistant bacterial strains of animal origin may spread into the human population by direct contacts and through food from animal sources. These resistant strains colonize the human intestine; and the genes coding resistance to antibiotics can be transferred to bacterial strains that belong to natural microflora (Bogaard and Stobberingh, 2000). The relation between the application of antibiotics and the dissemination of bacterial resistance from animals to humans was described by Hummel *et al.* (1986). Levey *et al.* (1976) also confirmed that in chickens fed with tetracycline there was a transfer of tetracycline resistance genes between chicken *Escherichia coli* strains, from chicken to chicken and from chicken to man.

An unfavorable factor in our study is to be seen in the increased frequency of ofloxacin- and ciprofloxacin-resistant strains of *Escherichia coli*. Bogaard and Stobberingh (2000) described 100% susceptibility of *Escherichia coli* strains to ciprofloxacin in Swedish and Dutch faecal samples of pigs. The high level of bacterial resistance to fluoroquinolones may be influenced by repeated administration of enrofloxacin, used for two years on the poultry farm in P., where ofloxacin- and ciprofloxacin-resistant strains of *Escherichia coli* were isolated. In enterococci, increased resistance to tetracycline, erythromycin and nitrofurantoin was registered. Higher occurrence of erythromycin-resistant strains was also proved in staphylococci. Besides the high resistance of staphylococci to erythromycin, the finding of an increased occurrence of clindamycin- and ofloxacin-resistant strains of *Staphylococcus* sp. is of importance too. The results can be causally related with the application of antimicrobial agents and should be further monitored.

In our study, *Enterococcus faecium* VanA strains were divided into two clusters (cluster 1 and 2) with 64% of reciprocal similarity. Within these clusters, the similarity of strains is high, therefore they are homogeneous (93–100%). It can be supposed that VanA strains in each of the two clusters are clonally related. On the contrary, strains susceptible to vancomycin (cluster 3) are highly heterogeneous. *Enterococcus faecium* VanB

included in cluster 1 can be considered as non-related with *Enterococcus* sp. group III VanB. Despite the fact that the value of plasmid profiling in VRE is questionable (Woodford *et al.*, 1998; Morrison *et al.*, 1999; Bopp *et al.*, 1999; Reineert *et al.*, 1999; Aarestrup, 2000), in our study the analysis of plasmid profiles confirmed PFGE delineation of isolates into genetically related groups and showed to be a useful supplementary typing method.

VanA gene cluster was originally localized on the nonconjugative plasmid pIP816 in *E. faecium* BM4147. Further investigations showed that these genes are a part of Tn 1546, a 10.8-kb transposon that carries the *vanRSHAXYZ* genes (Arthur *et al.*, 1993). Recent epidemiological studies of dissemination of VanA-type resistance in enterococci indicated the localization of the *vanA* gene cluster on 34- or 60-kbp plasmids (Clark *et al.*, 1993; Mato *et al.*, 1996), on conjugative >100-kbp plasmid (Werner *et al.*, 1999) or on the chromosome of bacteria. The *vanB* gene is located on large conjugative chromosomal elements or on plasmids (from 90 to 250 kbp). Recently, the presence of genes conferring VanB type vancomycin resistance in *E. faecalis* was shown to be on the 64-kbp composite transposon Tn1547 (Quintiliani and Courvalin, 1996). In strains *E. faecium* VanA 1, *E. faecium* VanA 2, and *E. faecium* VanA 3 we detected a plasmid >40 kbp in size, it is possible that these plasmids carry VanA-type resistance genes. The molecular sizes of plasmids detected in the other strains in our work (1.6 to 21 kbp) differ from the sizes of plasmids coding for either *vanA* or *vanB* reported by the above authors, therefore we propose chromosomal location of vancomycin resistance in these strains.

Considering VRE isolated from poultry and other animal sources, VanA type resistance is predominant (Stobberingh *et al.*, 1999; Bogaard *et al.*, 2002; Chen *et al.*, 2002). VanB type resistance (together with VanA type) in VRE from poultry products was also observed (Van den Braak *et al.*, 1998). Unlike to our findings, VanC type resistance was also found in VRE from poultry and pork (Lemck and Bulte, 2000). *VanC1*, *vanC2*, *vanC3* together with *vanA* gene were identified in *Enterococcus* strains, but no *vanB* gene was detected in these isolates. A possible relation between plasmid presence and/or plasmid profile and antibiotic resistance would be of great importance.

The results of this study confirm an increased level of resistance to some antibiotics in bacterial strains from poultry breeds and underline the importance of antibiotic policy implementation in veterinary medicine, including monitoring of bacterial strains with dangerous phenotypes of resistance. Monitoring of vancomycin resistance genes and their transfer in both animal and human strains is essential to obtain reliable data on the possibility of transmission of vancomycin resistance from animals to humans.

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