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Original article

INVESTIGATIONS ON TETRACYCLINE RESISTANCE IN COMMENSAL *ESCHERICHIA COLI* ISOLATES FROM SWINE

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Summary

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The distribution of tetracycline resistance in commensal *E. coli* strains, isolated from pigs at different stages of production system was investigated in four Bulgarian swine farms. The prevalence of antibiotic resistance and particularly tetracycline resistance, as well as two tetracycline resistance genes were evaluated in *Escherichia coli* isolates from swine faeces and manure lagoons. A total of 109 *E. coli* isolates from 116 faecal samples and 7 samples from manure lagoons were tested by disk diffusion method to determine resistance patterns to 10 antimicrobial agents. Tetracycline resistance was determined by disk diffusion method, micro-broth dilution method and qPCR. About 83% of the *E. coli* isolates from swine were resistant to one or more antimicrobial agents, respectively. The highest resistance observed in swine *E. coli* isolates was that to tetracycline (75.2%). The resistant *E. coli* isolates to tetracycline were examined for the presence of *tet* genes: *tet* (A) and *tet* (B). The most commonly identified *tet* gene was *tet* (A), which was found in 96.4% of swine and manure lagoon isolates.

Key words: commensal *Escherichia coli*, pig manure, tetracycline resistance

INTRODUCTION

Animal production farms are an important source for spread of genes coding for resistance to antibiotics in the environment. Consequently to the wide use of antibiotics in livestock production for both prevention and therapy, an increasing prevalence of various resistance genes in the environment is reported (Chee-Sandford *et al*., 2001; Kim *et al*., 2011; Li *et al*., 2013). According to Esiobu *et al.* (2002), there is a relationship between the use of antibiotics in pig farms and the occurrence of resistance genes in bacteria from manure, manure lagoons, as well as soil bacteria. On the other hand, the presence of resistant bacteria among the commensal microflora in organic cattle and pig farms, where the use of chemotherapeutics is limited, is also discussed (Hoyle *et al*., 2006; Jindal *et al*., 2012). This probably implies the existence of factors other than the selective pressure of chemotherapeutics to influence these processes.

Tetracyclines are often used in swine for therapy of bacterial enterites, gynaecological and respiratory infections, atrophic rhinitis etc. The variety of tetracycline resistance genes is commonly seen among both Gram-positive and Gram-negative bacteria, and their spread also refers to some specific environmental locations such as manure lagoons and underground waters (Callens *et al*., 2012; Jensen *et al*., 2012). Therefore, the tetracycline resistance could be used as a key factor in the monitoring of resistance genes in indicator porcine coli bacteria in studies on manure lagoons, underground waters and soils.

The horizontal transfer of resistance genes and in particular, tetracycline resistance genes is the main mechanism of their rapid spread among various bacterial species (Aminov *et al*., 2001; Chopra & Roberts, 2001). The manure from pig farms used for fertilisation of cultivable land, and faeces-contaminated underground waters could be regarded as critical zones for horizontal transfer of resistance genes and opportunistic bacterial species (Chee-Sanford *et al*., 2009). On the other side, the commensal intestinal microflora of domestic livestock is the main reservoir for exchange of genetic resistance determinants and that is why they are used for evaluation of antibiotic resistance levels in a number of European monitoring programmes along with zoonotic bacterial agents (WHO, 1997; Witte, 2000; Blake *et al*., 2003; Lappierre *et al*., 2008).

MATERIALS AND METHODS

Farms and antibiotic use policy

Farm – I
Total number of sows – 1100;
Number of manure lagoons – 1;
Antibiotic use policy: Wide use of colistin sulfate for metaphylaxis of post weaning enterites, etiologically associated with EHEC and ETEC in growing pigs. Wide use of amoxicillin and ceftiofur in various clinical forms of *S. suis* infection in suckling and growing pigs. The farm is free of dysentery and colonic spirochaetosis, which does not require the application of tiamulins and tetracyclines.

Farm – II
Total number of sows – 1180;
Number of manure lagoons – 1;
Antibiotic use policy: Wide use of colistin sulfate for metaphylaxis of post weaning enterites, etiologically associated with EHEC and ETEC in growing pigs. Due to the stationary nature of swine dysentery and proliferative enteropathy, a continuous use of tiamulin preparations and tetracyclines as well as tylosin is noted. Lincomycin and lincospectin are also commonly used.

Farm – III
Total number of sows – 4000;
Number of manure lagoons – 1;
Antibiotic use policy: Wide use of ceftiofur for metaphylaxis of streptococcal infections in suckling and growing pigs, as well as administration of tetracyclines, lincomycin and amoxicillin.

Farm – IV
Total number of sows – 1500;
Number of manure lagoons – 1;
Antibiotic use policy: Wide use of ceftiofur for metaphylaxis of streptococcal infections in suckling and growing pigs. Due to the stationary nature of swine dysentery and colonic spirochaetosis, lincospectin, tiamulins often combined with oxy- or chlortetracycline are used.

Collection of the samples

Between December 2013 and May 2014, 120 faecal swab samples were collected

from different age groups of pigs (suckling, weaned, finisher) as well as from manure lagoons at farms. Faecal swabs were transported in Stuart Transport Medium (BD, USA) at low temperature within 18–24 hours. The materials for microbiological examination from each of manure lagoons were collected from the liquid phase at a depth of 20–40 cm. The total sample volume consisted of 3 separate samples with equal volume, which were transported in sterile containers at low temperature.

Culturing and identification of E. coli isolates

Swab and lagoon samples were cultured on Mc Conkey agar (Emapol, Poland) at 37 ^{o}C for 24 hours. Lactose-positive colonies were sub-cultured onto triple-sugar iron (TSI) agar (BD, USA) and submitted to preliminary biochemical typing via citrate utilisation, methyl red, Vogues Proskauer and indole production tests. The identification of strains was performed with kits for non-fermenting and enteric bacteria (BD, USA) and the semi-automated Crystal BBL identification system.

Determination of the sensitivity of E. coli isolates to antibiotics

The sensitivity of *E. coli* isolates to 10 chemotherapeutics was evaluated by the disk diffusion method as per CLSI (2010), using Muller-Hinton agar (Emapol, Poland) and antibiotic disks (Emapol, Poland), loaded as followed: amoxicillin (10 μg), cephalotin (30 μg), ceftazidime (10 μg), cefotaxime (30 μg), gentamicin (10 μg), streptomycin (10 μg), spectinomycin (25 μg), tetracycline (30 μg), enrofloxacin (5 μg), sulfamethoxazole (25 μg). To determine the sensitivity of isolates to beta lactams, amoxicillin, cephalotin, cefotaxime and ceftazidime, the synergic amoxicillin/clavulanic acid (20/10 μg) test was used. Antibiogrammes were controlled with a reference strain *Escherichia coli* ATTC 25922.

The tetracycline MIC were determined with micro-broth dilution test and cation-adjusted Muller-Hinton broth (Emapol, Poland), by preparation of doubling dilutions of tetracycline within 0.25–32 μg/mL. The tetracycline resistance breakpoint was ≥ 16 μg/mL.

Determination of tetracycline resistance genes in E. coli strains

DNA extraction. For DNA extraction, 24-hour cultures incubated at 37 ^{o}C, respectively 3–4 colonies on McConkey agar were suspended in 100 μL sterile distilled water free of inhibitors for molecular diagnostics (Qiagen, Germany). The DNA extraction kit DNeasy Blood Tissue Kit (Qiagen, Germany) was carried out.

Amplification method. To determine the tetracycline resistance genes, Microbial DNA qPCR Assay, *tet* (A) and *tet* (B) (Qiagen, Germany) were used. The qPCR amplification was done with Stratagene Mx3000P instrument. The thermocycler protocol consisted of: initial PCR activation 10 min, 95^{o}C, 1 cycle, 2-step cycling – denaturation (15 s, 95 ^{o}C), annealing and extension (2 min, 60 ^{o}C ×40 cycles). The results were interpreted according to manufacturer's instructions (negative control signal at C_T<35 and C_T= 22±2 for internal positive PCR control, PPC).

RESULTS

The total number of *E. coli* isolates from examined swab samples was 102, and from manure lagoons – 7.

Data about the presence of resistant *E. coli* strains isolated from the different

categories of pigs and lagoons at the four farms are summarised in Table 1. The highest number of resistant strains to the 10 tested chemotherapeutics was found in the groups of weaned pigs – 40.5%, followed by finisher pigs with 23.8% and the neonatal group with 15.5%. The total prevalence of resistant *E. coli* isolates for the four farms was 83.4%.

The resistance patterns of *E. coli* strains in the different age groups at the farms are presented in Table 2. A total of 18 resistance patterns were observed. The Ax S SPT T SMZ resistance pattern exhibited the highest percentage (28.4%) of multi-resistant strains, followed by the Ax S SPT T with 19.3%. Tetracycline belonged to the 15 identified resistance

Table 1. Prevalence of resistance to at least one of 10 tested antimicrobials among faecal *E. coli* from pigs on 4 farrow-to-finish farms

Source	Prevalence of resistance (number/%)	95% confidence limits
Suckling pigs (n=24)	17/15.5%	9.0÷22.4
Weaned pigs (n=49)	44/40.5 %	31.4÷49.9
Finisher pigs (n=29)	26/23.8%	16.1÷31.9
Manure lagoon (n=7)	4/3.7%	0.9÷8.0
Total (n=109)	91/83.4%	75.3÷89.4

Table 2. Patterns of resistance to 10 antimicrobials among *E. coli* from 4 farrow-to-finish farms (n=91)

Patterns of resistance to 10 antimicrobials	Number of resistant isolates	Percentage of resistant isolates
Ax KF S SPT T Enr	1	1.1
Ax KF GN S SPT T	2	2.3
Ax GN S SPT T SMZ	4	4.5
Ax S SPT T Enr	1	1.1
Ax KF S SPT T	4	4.5
Ax GN S SPT T	2	2.3
Ax KF GN S T	5	1.1
GN S SPT T SMZ	2	2.3
Ax S SPT T SMZ	25	28.4
Ax KF T SMZ	1	1.1
Ax KF T	4	4.5
Ax S SPT SMZ	4	4.5
Ax S SPT T	17	19.3
GN S SPT T	3	3.4
Ax KF	1	1.1
S SPT T	4	4.5
GN S SPT	4	4.5
T	7	7.9

Ax–amoxicillin; KF–cephalotin; CAZ–ceftazidime; CTX–cefotaxime; GN–gentamicin; S–streptomycin; SPT–spectinomycin; T–tetracycline; Enr–enrofloxacin; SMZ–sulfamethoxazole.

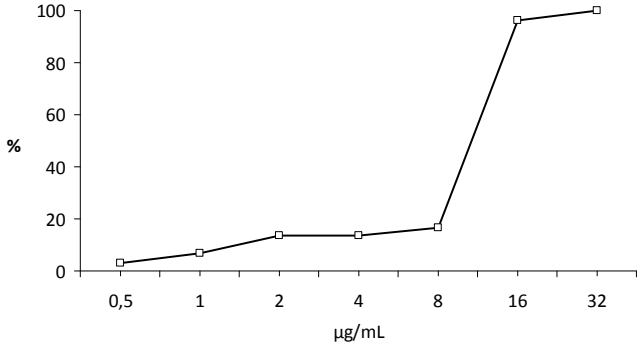

Fig. 1. MIC of tetracycline in *E. coli* isolates from pigs.

Table 3. Detected resistance genes in phenotypically tetracycline resistant porcine *E. coli* (n=91)

Genotype	Number/percentage					
	Suckling pigs (n=24)	Weaned pigs (n=49)	Finishers (n=29)	Manure lagoon (n=7)	Total (n=109)	CL 95%
Tetracycline resistance	17/15.5%	44/40.5%	26/23.8%	4/3.7%	91/83.4%	75.3÷89.4
tet (A)	14/12.8%	41/37.6%	21/19.2%	3/2.7%	79/72.4%	63.7÷80.3
tet (B)	–	–	2/1.8%	1/0.9%	3/2.7%	0.5÷6.5
tet (A) *tet* (B)	–	–	–	–	–	

CL – confidence limits.

patterns; resistance to tetracycline only was demonstrated by 7.9% of isolates.

The cumulative curve of tetracycline on the basis of detected MICs of tetracycline in tested strains (Fig. 1) showed that the MIC_{90} value was 16 µg/mL, and two of resistant isolates had MICs of 32 µg/mL.

The occurrence of efflux genes *tet* (A) and *tet* (B) in *E. coli* strains isolated from pigs at different ages and from manure lagoons at surveyed farms are presented in Table 3. The prevalence of gene *tet* (A) among strains isolated from pigs and lagoons was 72.4%. Two of *E. coli* strains from the finisher pigs group (1.8%) and 1 isolate from the lagoons (0.9%) possessed *tet* (B). None of tetracycline-resistant strains in this study exhibited the combination of *tet* (A) and *tet* (B).

DISCUSSION

Literature data with information about the role of manure utilisation and the spread of resistance genes in animal farm environment, the soil and underground waters are few. Therefore, studies reporting such data would be useful allowing a more objective evaluation of the risk from using chemotherapeutics in animal production systems. From this point of view, an important fact which sometimes precludes the considerable difference in reports from European and North America researchers is that in the USA and Canada,

chemotherapeutics including tetracyclines are still used not only for therapy but also as growth promoters.

Barton (2000) and Teuber (2001) affirm that in animal practice, about 50% of chemotherapeutics are used at subtherapeutic doses for prophylaxis and as growth promoters. Data of the American Health Institute from 2001 place tetracyclines on the leading place as their use in medicine is concerned: 3,239 tonnes annually, followed by macrolides, lincosamides, polypeptides, streptogramins and cephalosporins, 1,937 tonnes per year. In Denmark, Aarestrup (2005) determined tetracyclines as the most commonly used antibiotics in the animal practice. In 2009, according to data of European Medicines Agency (2011) and Grave *et al*. (2012), the share of tetracyclines in sales of antibiotics for use in animals was 40%. These facts certainly uncover the risk posed by the high selective pressure on the spread of resistance to chemotherapeutics in men, animals and the environment. In addition, the great diversity of bacterial species isolated from the manure of pig farms – 60 belonging to 28 genera, acknowledged to possess tetracycline resistance and plasmid factors for transfer of genetic determinants, should also be taken into consideration (Binh *et al*., 2008).

The results from the European project "Antibiotic resistance in bacteria of animal origin -II" (ARBAO-II) from 2004, regarding the sensitivity to tetracycline of porcine commensal *E. coli* strains indicate a high percentage of resistance in a number of EC countries: 95.6% in Poland, 86.0% in Great Britain, 81.0% in France, 63.9% in Finland, 58.1% in Austria, 43.8% in Denmark and 31.9% in the Netherlands (Hendriksen *et al*., 2008). In Bulgaria, such long-term monitoring studies on the resistance to antibiotics in commensal enteric bacteria have not been carried out and our data obtained from four large pig farms in the country, indicating a high prevalence of resistance (83.4%) among porcine *E. coli* commensals, could be commented in the light of aforementioned data.

Akwar *et al*. (2008) performed an analysis of the high prevalence of tetracycline resistance (89.5%) in *E. coli* strains in healthy weaned pigs, which is explained with the fact that this technological group is highly sensitive to bacterial infections and thus, more frequently subjected to therapeutic antibiotic pressure than finisher pigs. The phenotypic as well as genotypic expression of tetracycline resistance shown in this study is also with highest percentage (40.5%; 37.6%) in *E. coli* strains isolated from weaned pigs.

Blake *et al*. (2003) discussed the presence of *tet* (B) and high MIC in *E. coli* strains from pigs with multi-resistance profiles including ampicillin, streptomycin and chloramphenicol. According to the researchers, *tet* (A) and *tet* (C) are detected in strains with lower resistance levels as determined by MIC, moreover, they believe that these genes are not present in isolates from intensive pig farms. They have neither found more than one gene in tetracycline-resistant *E. coli* strains unlike Marshall *et al*. (1983) and Lee *et al*. (2000), which in their opinion had not any significant effect on MICs. Lanz *et al*. (2003) report a high occurrence of 87% of *tet* (A) in porcine clinical tetracycline-resistant *E. coli* strains. Sengeløv *et al*. (2003) also discussed the dominance of *tet* (A) in *E. coli* strains from healthy and diseased pigs, cattle and poultry (71%) and did not establish any difference in the prevalence of resistance genes in commensal and clinical strains. The association of high MIC to tetracyc-

line and occurrence of *tet* (B) are also commented by Lee *et al*. (2000) and Sunde *et al*. (1998). Lee *et al.* (1993) demonstrated MIC >128 μg/mL in 93% of resistant *E. coli* bacteria from pigs with *tet* (B). In the USA, Bryan *et al*. (2004) established a high level of tetracycline resistance of 78% in commensal coli bacteria from pigs, at the background of high MIC – 233 μg/mL, the genotype of which was related to higher percentage of *tet* (B) than of *tet* (A): 63% vs 35% respectively. In 30% of isolates the authors observed more than one gene coding for tetracycline resistance. The established MIC_{90} to tetracycline was 16 μg/mL, in *E. coli* isolates from the different age groups and manure lagoons, and the value is comparable to that commented by Blake *et al*. (2003), with the only difference that our survey was performed in intensive pig farms.

In the Republic of Korea, Cho & Kim (2008) discussed the high occurrence (90.3%) of tetracycline resistance in commensal *E. coli* strains isolated from pigs with involvement of the *tet* (A) genotype in 98.2% of cases. In Canada, Kozac *et al*. (2009) also observed a high tetracycline resistance of 83% in commensal porcine *E. coli* strains, although the more common genotypic expression was that of *tet* (B) – 59%. The monitoring of commensal *E. coli* strains from pigs carrying the tetracycline resistance genes conducted by Schwaiger *et al.* (2010) has shown a predominance with >55% of *tet* (A) and the presence of a single gene only in 88% of cases.

In our study, the genotypic expression of tetracycline resistance was characterised with dominance of *tet* (A) in 72.4% of resistant isolates, so our results corresponded the best to those of Sengelőv *et al*. (2003) and Cho & Kim (2008).

Barkovskii & Bridges (2012) found out preponderance of genes determining the mechanism of ribosomal protection *tet* (M) as well as efflux genes. The authors outlined the close association between resistance genes from animal faeces and those in lagoons, and concluded that lagoons were an important source of resistance genes in the farm environment. They discussed the thesis that *tet* (B) could be determined as indicator gene in both faeces and manure lagoons at farms utilising chlortetracycline as growth promoter. The more general analysis of the three studied farms from the cited study however allowed affirming that the diversity of genetic determinants at pig farms was not dependent on the use of tetracycline. In *E. coli* isolates from lagoons and other facilities in the proximity of pig farms, Graves *et al*. (2011) established a high incidence of 91.3% of *tet* (A) and *tet* (B). With respect to the spread of genetic determinants of resistance in *E. coli* isolates from the lagoons of studied farms, we proved a higher occurrence of *tet* (A) – 2.7% than of *tet* (B) – 0.9%. Analysing the data about manure lagoons isolates, it should be said that first, they were few and second, that four of them exhibited tetracycline resistance.

CONCLUSION

Antimicrobial resistance of commensal *E. coli* is of public health significance, particularly the quick selection of multidrug-resistant populations in different group of animals. The horizontal gene transfer of commensal *E. coli* can be associated to co-resistance and may be transferred to pathogenic strains of *Enterobacteriaceae* spp. There are several examples of humans colonised by resistant commensal *E. coli* from food animals and there are pos-

sibilities for limiting therapeutic options. That is why the monitoring of antimicrobial resistances in commensal *E. coli* from food animals and the farm environment is important for real trends assessment.

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