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INVESTIGATIONS ON THE RESISTANCE OF COMMENSAL SWINE *ESCHERICHIA COLI* TO SOME AMINOGLYCOSIDES-AMINOCYCLITOLS

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Abstract

The aim of this study was to describe the prevalence of antibiotic resistance to some aminoglycosides, streptomycin, spectinomycin and gentamicin and three aminoglycoside- resistance genes in *Escherichia coli* isolated from feces and lagoon manure in six swine farms in Republic of Bulgaria. A total of 274 *E. coli* isolates from 270 fecal samples and twelve samples from lagoon manure were tested by disk diffusion method to determine resistance patterns to 11 antimicrobial agents. Aminoglycosides resistance also was determined by E-test, agar dilution method, PCR and qPCR. The highest resistance observed to streptomycin (70.0%) and spectinomycin (65.5%). Multi-resistance patterns in studied *E. coli* strains showed that the resistance to streptomycin/spectinomycin was most frequently seen together with resistance to ampicillin, tetracycline, and sulfonamides (39.6%). The *E. coli* isolates resistant to streptomycin, spectinomycin were examined for the presence of *strA/strB, aadA1* genes, and resistant isolates to gentamicin were evaluated for the presence of the *aacC1* gene. The most common gene determining resistance to aminoglycosides was *aadA1* which was found in 54.0% of swine isolates and lagoon manure isolates followed by *straA/strB* genes (32.3%). The *aacC1*gene was not identified in *E. coli* isolates resistant to gentamicin.

Keywords: aminoglycoside resistance, commensal *Escherichia coli*, pigs, lagoon manure

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ISPITIVANJE REZISTENCIJE KOMENSALNE *ESCHERICHIA COLI* KOD SVINJA NA NEKE AMINOGLIKOZIDE-AMINOCIKLITOLE

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Kratki sadržaj

Cilj ovog istraživanja je prikaz prevalencije rezistentnosti na antibiotike i to neke aminoglikozide, streptomicin, spektinomicin i gentamicin kao i tri gena *Escherichia coli* koji uslovljavaju rezistenciju na aminoglikozide , izolovanih iz fecesa i osoke iz sabirnih bazena na 6 farmi svinja u Republici Bugarskoj. Ispitano je 274 izolata *E. coli* dobijenih iz 270 uzoraka fecesa i 12 uzoraka osoke. Uzorci su testirani metodom disk difuzije u cilju odredjivanja modela rezistencije na 11 antimikrobnih agenasa. Rezistencija na aminoglikozide je takodje odredjivana primenom E-testa, metode dilucije agara, PCR i qPCR. Najveći stepen rezistencije ustanovljen je za streptomicin (70.0%) i spektinomicin (65.5%). Modeli višestruke rezistencije uočeni kod sojeva *E. coli* pokazali su da se rezistencija na streptomicin/spektinomicin najčešće javlja udružena sa rezistencijom na ampicilin, tetraciklin i sulfonamide (39.6%). Izolati *E. coli* rezistentni na streptomicin i spektinomicin ispitani su na prisustvo gena *strA/strB, aadA1*, a izolati rezistentni na gentamicin na prisustvo gena *aacC1*. Ustanovljeno je da je rezistencija na aminoglikozide najčešće bila uslovljena genom *aadA1* koji je detektovan kod 54.0% izolata svinja i izolata osoke iz sabirnih bazena, a nakon njega slede geni *straA/strB* (32.3%). Ben *aacC1* nije identifikovan kod izolata *E. coli* rezistentnih na gentamicin.

Ključne reči: rezistencija na aminoglikozide, komensalna *Escherichia coli*, svinje, osoka iz sabirnih bazena

INTRODUCTION

The concept of EMA (2014) on the use of aminoglycosides in livestock and companion animals in the EU, development of resistance and public health risks is grounded on data about the increasing resistance to aminoglycosides in animal and human bacterial isolates. Data of EMA/ESVAC (2013) demonstra-

te a widespread use of aminoglycosides in some animal species in particular (large and small ruminants, swine, horses, and pets) for treatment of septicaemic states, gastrointestinal infections, respiratory and urogenital infections. Some authors discussed the potential of resistant *E. coli* from domestic animals as a reservoir for the spread of resistance to aminoglycoside antibiotics among human population (Chaslus-Dancla et al., 1991; Jonson et al., 1994, 1995). The primary mechanism of aminoglycoside resistance is the production of aminoglycoside modifying enzymes. Three genetic determinants are associated to the expression of resistance to streptomycin in enterobacteria: *ant(3″)-Ia* (synonym *aadA*) coding for the production of adenyltransferase ANT (3″)-I, modifying streptomycin and spectinomycin, *aph(3″) –Ib* (synonym *strA*), determining the production of the phosphoryltransferase APH (3″)-I, modifying streptomycin and *aph(6)-Id* (synonym *strB*), responsible for the production of phosphoryltransferase APH (6)-I, that also modifies streptomycin (Heinzel et al., 1988; Hollingshead and Vapnek, 1985; Scholz et al., 1989). During the last years, reports about a new type of aminoglycoside resistance in animal bacterial isolates related to the prevalence of 16S rRNA methylases and the respective high levels of resistance are increasing (Gonzalez-Zorn et al., 2005; Chen et al., 2007; Liu et al., 2008; Du et al., 2009; Davis et al., 2010; Hopkins et al., 2010; Deng et al., 2011). From modifying enzymes coding for resistance to gentamicin in E. coli strains from livestock, adenyltransferase ACC(3) – IV whose production is coded by the *aac3-IV* gene and that determines a combined resistance to gentamicin and apramycin, is of special interest. There are also data, although limited, on the prevalence of *aacC1* and *aac3-II* genes in domestic animals (Guerra et al., 2003; Sãenz et al., 2004). These genes determine the production of acetyltransferases ACC (3)- I and AAC (3)-II, distinguished with their phenotype profile, which for the latter gene includes also resistance to tobramycin apart to gentamicin (Vaculenko and Mobashery, 2003).

MATERIAL AND METHODS

Sample collection

Between January 2013 and September 2014, 282 faecal swab samples were collected from different age groups of pigs (suckling, weaned, finisher) and lagoon manure from 6 farrow-to-finish farms. Faecal swabs were transported in Stuart Transport Medium (BD, USA) at low temperature within 18-24 hours.

Culturing and identification of E. coli isolates

Swabs were cultured on McConkey agar (Emapol, Poland) at 37 °C for 24

hours. Lactose-positive colonies were subcultured onto 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 identification Crystal BBL system.

Determination of the sensitivity of E. coli isolates to antibiotics

The sensitivity of *E. coli* isolates to 11 chemotherapeutics was evaluated by the disk diffusion method as per CLSI, using Muller-Hinton agar (Emapol, Poland) and antibiotic disks (Emapol, Poland), loaded as followed: ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cephalotin (30 μg), ceftazidime (10 μg), cefotaxime (30 μg), gentamicin (10 μg), streptomycin (10 μg), spectinomycin (25 μg), tetracycline (30 μg), ciprofloxacin (5 μg), sulfamethoxazole (25 μg). The reference strain *Escherichia coli* ATTC 25922 was used for the control of disk diffusion method and MIC method.

The streptomycin MIC was determined in the agar dilution test and Muller-Hinton agar (Emapol, Poland), by preparation of doubling dilutions of streptomycin (Sigma-Aldrich) within 0.01-256 μg/mL. MIC for gentamicin was defined by E test strips (AB Biodisk, Solna Sweden). MICs were interpreted according to epidemiological criteria (EUCAST, www.eucast.org).

Determination of resistance genes to aminoglycoside in commensal E. coli

DNA extraction: For DNA extraction, 24-hour cultures incubated at 37°C, respectively 3-4 colonies on McConkey agar were suspended in 100 μl sterile distilled water free of inhibitors for molecular diagnostics (Qiagen). The DNA extraction kit DNeasy Blood Tissue Kit (Qiagen) was used.

Detection of resistance genes: The presence of resistance genes to aminoglycoside antibiotics, *aadA1* was detected by qPCR and *strA/strB* by PCR. The primers sequences for *strA/strB* were strA–F ATGGTGGACCCTAAAACTCT and strB-R CGTCTAGGATCGAGACAAAG (Kozak et al., 2009). PCR assays in 25 μl final volume contained 12.5 μl Taq PCR Master mix (Qiagen) and 3 μl DNA template. The PCR reaction for *strA/strB* consisted of an initial activation step at 94°C for 10 min, followed by 30 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 63°C for 1 min, and primer extension at 72°C for 30 s, and final extension for 10 min at 72°C. All reactions were carried out in Eppendorf gradient thermal cycler. Ten μL aliquots of PCR products were analyzed by gel electrophoresis with 1.5% agarose gel (Peqlab, Germany). Gels were stained with ethidium bromide at concentration 10 μg/mL and visualized by UV transillumination. A 100 bp DNA ladder plus (Qiagen) was used as marker. As positive control strain *E. coli* 94.4 was used, provided by Mrs. J. Mazurec from

the Department of Molecular Biology, Faculty of Biological Sciences, University of Zielona Góra, Poland. Negative controls were PCR mixtures with the addition of water in place of template DNA.

To determine the *aadA1*gene, Microbial DNA qPCR Assay, *aadA1* (Qiagen) was used. qPCR amplification was done with Stratagene Mx3000P instrument. The thermocycler protocol consisted of: initial PCR activation 1 cycle of 10 min at 95°C, and 40 cycles of 2-step cycling – denaturation - 15 sec 95°C, annealing and extension 2 min 60°C. The results were interpreted according to manufacturer's instructions (negative control signal at CT>35 and CT= 22±2 for positive control).

RESULTS

Number of isolates: The total number of *E. coli* isolates from examined faecal swabs obtained from the different age categories and from lagoon manure at studied farms were 274.

Prevalence of antibiotic resistance by disk-diffusion method

Tables 1, 2 and 3 present results from the phenotype analysis of resistance of *E. coli* isolates from the 6 surveyed farms to 11 chemotherapeutics. With respect to aminoglycosides, the highest resistance percentages to streptomycin and spectinomycin (93.2% and 91.0 % respectively) were observed in finisher pigs. Higher resistance to gentamicin (15.7%) was established in *E. coli* isolates from weaned pigs than in finisher (7.9%) or neonatal pigs (4.5%).

Table1. Prevalence of antibiotic resistance in *E. coli* strains from suckling pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=14	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ampicillin	1 (6.7)	3 (21.4)	6 (40.0)*	3 (20.0)	2 (13.3)	2 (13.3)	17 (19.0.)
Amoxicillin/ clavulanic acid	0	0	0	0	0	0	0
Cephalotin	0	1 (7.1)	3 (20.0)	0	0	0	4 (4.5)

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=14	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	1 (7.1)	3 (20.0)	0	0	0	4 (4.5)
Strep-tomycin	1 (6.7)	3 (21.4)	9 (60.0)**	3 (20.0)	4 (26.6)	2 (13.3)	22 (24.7)
Specti-nomycin	0	2 (14.3)	7 (46.6)*	2 (13.3)	4 (26.6)	0	15 (16.8)
Tetracycline	3 (20.0)	9 (64.3)**	12 (80.0)***	10 (66.6)**	5 (33.3)	8 (53.3)*	47 (52.8)
Cipro-floxacin	0	1 (7.1)	1 (6.7)	0	0	0	2 (2.2)
Sulfamet-hoxazole	2 (13.3)	3 (21.4)	5 (33.3)	3 (20.0)	3 (20.0)	2 (13.3)	18 (20.2)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

Data about the resistance of *E. coli* isolates to aminoglycosides in groups of suckling pigs showed the highest resistance to streptomycin, spectinomycin and gentamicin (60%, 46.6%, and 20% respectively) at farm 3.

Isolates resistant to streptomycin and spectinomycin were the most prevalent (100%) among weaned pigs from farms 3 and 5, followed by the occurrence of resistance to aminoglycosides in 86.6% of isolates from farms 4 and 6 and in 85.7% of isolates from farms 1 and 2. The percentage of isolates from weaned pigs resistant to gentamicin was the highest at farm 4 (7.8%).

Table 2. Prevalence of antibiotic resistance in *E. coli* strains from weaned pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=14	Farm II n=15	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ampicillin	3 (21.4)	10 (66.6)**	15 (100)***	14 (93.3)***	12 (13.3)***	6 (40.0)	60 (67.4)
Amoxicillin/ clavulanic acid	0	0	2 (13.3)	0	0	0	2 (2.2)
Cephalotin	0	4 (26.6)	12 (80.0)***	10 (66.6)***	2 (13.3)	4 (26.6)	32 (35.9)
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	3 (20.0)	4 (26.6)	7 (46.6)	0	0	14 (15.7)
Streptomycin	12 (85.7)	12 (85.7)	15 (100)*	13 (86.6)	15 (100)*	13 (86.6)	80 (89.8)
Spectinomycin	12 (85.7)	12 (85.7)	15 (100)*	13 (86.6)	15 (100)*	13 (86.6)	80 (89.8)
Tetracycline	10 (71.4)	15 (100)**	15 (100)**	11 (73.3)	14 (93.3)**	11 (73.3)	76 (85.3)
Ciprofloxacin	0	2 (13.3)	3 (20.0)	0	1 (6.7)	0	6 (6.7)
Sulfamethoxazole	5 (35.7)	14 (93.3)***	15 (100)***	11 (73.3)*	15 (100)***	12 (80.0)**	72 (80.8)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

The highest resistance percentage to streptomycin and spectinomycin (100%) was demonstrated in *E. coli* isolates from finisher pigs at farms 2, 3, 4 and 5. With respect to gentamicin, the highest resistance (28.5%) was found out among isolates from farm 3.

Table 3. Prevalence of antibiotic resistance in *E. coli* strains from finishers pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=15	Farm III n=14	Farm IV n=14	Farm V n=15	Farm VI n=15	Total n=88
Ampicillin	1 (6.7)	3 (20.0)	4 (28.5)	3 (21.4)	3 (20.0)	4 (26.6)	18 (20.3)
Amoxi-cillin/ cla-vulanic acid	0	0	0	0	0	0	0
Cephalotin	0	3 (20.0)	6 (42.8)**	2 (14.2)	3 (20.0)	1 (6.7)	15 (17.0)
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	2 (13.3)	4 (28.5)	1 (7.1)	0	0	7 (7.9)
Strep-tomycin	10 (66.6)	15 (100)***	14 (100)***	14 (100)***	15 (100)***	14 (93.3)*	82 (93.2)
Specti-nomycin	9 (60.0)	15 (100)***	14 (100)***	13 (92.8)	15 (100)***	14 (93.3)*	80 (91.0)
Tetracycline	9 (60.0)	14 (93.3)*	14 (100)***	12 (85.7)	10 (66.6)	10 (66.6)	69 (78.2)
Cipro-floxacin	0	1 (6.7)	2 (14.2)	0	1 (6.7)	0	4 (4.5)
Sulfamet-hoxazole	4 (26.6)	8 (53.3)	13 (92.8)***	8 (57.1)	6 (40.0)	6 (40.0)	45 (51.1)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

Among multiresistant isolates, the highest prevalence of 23.6% was that of the phenotype profile including ampicillin, streptomycin, spectinomycin and tetracycline, following by the profiles of resistance to ampicillin, streptomycin, spectinomycin and sulfamethoxazole (9.7%) and to ampicillin, cephalotin, gentamicin, streptomycin and tetracycline (6.3%).

Phenotypic analysis of MIC concentrations for streptomycin and gentamicin

Table 4 presents the cumulative MIC percentages to streptomycin and gentamicin. The MIC90 of isolates to streptomycin was 16 μg/mL, whereas to gentamicin MIC90 was 1 μg/mL.

Table 4. Distribution of MICs among commensal *E. coli* (n=274) isolated from pigs and lagoon manure

Antibiotic	Cumulative (%) MIC in μg/mL												
	0.06	0.125	0.250	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128	≥256
Strep-tomycin			4.8	10.1	25.7	27.0	28.7	30.0	82.8	97.8	100		
Gentamicin	58.3	82.7	85.3	88.3	98.5	98.9	98.9	100					

Occurrence of resistance determinants

Table 5 presents the prevalence of resistance genes *strA/strB* and *aadA1* among *E. coli* isolates from different categories of pigs and manure lagoons resistant to streptomycin and spectinomycin. The highest occurrence (54.0%) was that of *aadA1* among isolates resistant to streptomycin and spectinomycin, whereas 32.3% of isolates were positive for *strA/strB*. The combination of *aadA1* and *strA/strB* genes was determined in 3.1% of strains. The analysis of data on the distribution of resistance genes among the different age categories, the highest prevalence of *aadA1* (23.4%) was observed in finisher pigs, while *strA/strB* genes were the most frequently encountered among weaned pigs. Isolates form suckling pigs also showed a higher prevalence of *aadA1* (7.8%). Higher prevalence of *aadA1* (2.1%) was established in *E. coli* isolates from manure lagoons as compared to *strA/strB* positive strains (1.0%). The combination of *aadA1* and *strA/strB* was observed in 3.2% of resistant strains; similar were percentages (1.5%, 1.0%) among isolates from weaned and finisher pigs. The same combination of resistance genes was not found out in isolates from suckling pigs.

None of E. coli strains resistant to gentamicin has exhibited the *aacC1* gene.

Table 5. Occurrence of resistance genes determined among commensal *E. coli* (n= 274) from pigs and lagoon manure

Occurrence [n (%)]						
Genotype	Suckling pigs	Weaned pigs	Finishers	Manure lagoon	Total	95%CL
Resistance to streptomycin (%)	22 (24.7)***	80 (89.8)***	82 (93.2.)***	8 (2.9)	192 (70)	60.1÷79.0
aadA1	15 (7.8)	40 (20.8)	45 (23.4)	4 (2.1)	104 (54.0)	46.9÷60.9
strA/strB	3 (1.5)	30 (15.6)	27 (14.1)	2 (1.0)	62 (32.3)	25.9÷39.0
aadA1+strA/strB	-	3 (1.5)	2 (1.0)	1 (0.5)	6 (3.1)	1.1÷6.0

Legend: p≤0.05 (*); p≤0. 01(**); p≤0.001 (***)

DISCUSSION

Molecular characteristics of the commonest co-resistant phenotypes in commensal *E. coli* isolates from domestic animals are related to the presence of *bla TEM-1* coding resistance to ampicillin, *aadA1* and strA/*strB* determining streptomycin resistance, *tet (A)* and *tet (B)* in tetracycline-resistant strains, *sul1* in sulfamethoxazole-resistant and *dfrA1* – in trimethoprim-resistant isolates. The resistance to gentamicin among commensal poultry and swine *E. coli* isolates is outlined with an ascending trend (Szmolka et al., 2013). The authors presented data from the Hungarian Antimicrobial Monitoring System, showing that the resistance to gentamicin among commensal porcine *E. coli* isolates kept the usual low levels throughout the monitoring period from 2004 to 2008. In their view, the prevalence of *strA*, *strB*, and *aadA1* genes among porcine *E. coli* strains was within the ranges 30%-60%, 60%-100%, and 1-30% respectively. Sundin et al. (1996) commented the wide spread of *strA/strB* genes in the environment and the relationship between their spread and exchange in the different ecological niches, plants, livestock animal species and humans. Sandvang et al. (2000) and Jakobsen et al. (2007) also discussed the incidence of *ant (2$^{//}$) –I*, *aac (3)-IIa*, and *aac(3)-IVa* among gentamicin-resistant *E. coli*

strains from pigs and other domestic animals. Guerra et al. (2003) presented the prevalence of *aadA1* (61%) and *strA/strB* (59%) resistance genes in commensal *E. coli* from poultry, swine and cattle. Mazurec et al. (2013) detected the presence of *aadA1* in 35.0% of streptomycin-resistant commensal porcine *E. coli* isolates.

Data about the resistance to streptomycin among commensal *E. coli* isolates from pigs show a substantial variability. For instance Wasyl et al. (2007) reported the presence of resistance in 37.0% commensal porcine *E. coli* isolates while streptomycin resistance among isolates from weaned animals reported by Stannarius et al. (2009) was 60.6%. The results of Mazurec et al. (2013) with respect to streptomycin resistance showed that it was present in 88.3% of tested commensal *E. coli* strains from swine.

The prevalence of resistance to streptomycin (70.0%) and spectinomycin (65.5%) among commensal porcine *E. coli* isolates in the present study was comparable to the results of Stannarius et al. (2009) and Mazurec et al. (2013). It should be noted that according to our data, the resistance to gentamicin (12.4%) was considerably higher that reported by Szmolka et al. (2012).

The percentage of *strA/strB* positive streptomycin-resistant strains in this study (32.3%) was the same as results presented by Szmolka and Nagy (2013), yet the occurrence of the *aadA1* gene (54.0%) was higher.

CONCLUSION

The established resistance to streptomycin and spectinomycin to commensal *E. coli* strains from pigs was close to the highest percentages reported by different research teams from the EU member states. As the genetic resistance profile was concerned, the prevalence of the *aadA1* genes was incontestable among isolates from the different age categories of pigs and environmental strains (from manure lagoons). The lack of *aacC1* genes from the genetic profile of resistance to gentamicin was not an exception as could be seen from limited data on their prevalence among domestic animals and at present, the data for the occurrence of these genes in commensal porcine *E. coli* strains are predominantly from the Asia region.

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