Prevalence of resistance to sulfonamides and streptomycin among commensal porcine *Escherichia coli* isolates

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**ABSTRACT**

The aim of this study was to describe the prevalence of antibiotic resistance to streptomycin, spectinomycin and sulfamethoxazole and resistance genes *aadA1*, *sul1*, *sul2*, *intI* in *Escherichia coli* isolates from faeces and lagoon manure on seven swine farms in the Republic of Bulgaria. A total of 540 *E. coli* isolates isolated from 574 faecal samples and samples from manure lagoons were tested by disk diffusion method to determine resistance patterns to 11 antimicrobial agents and by the MIC plate method for streptomycin and sulfamethoxazole. The highest resistance in swine *E.coli* isolates was observed to streptomycin – 69.4% and spectinomycin – 63.3%. Multi-resistance patterns in studied *E. coli* strains showed that the resistance to streptomycin/spectinomycin was most frequently seen together with resistance to tetracycline, and sulfonamides (27.8%). The *aadA1* gene was found in 54.4% of swine isolates and lagoon manure isolates. The *sul1* gene was identified in 20.3% and *sul2* gene in 21.2% of *E. coli* isolates. The prevalence of *intI* gene was determined in 9.8% of isolates. The presence of variable region of class I integrons with size 1 kb was found in 5.3% of *E.coli* isolates.

**Keywords:** streptomycin and sulfonamide resistance, commensal *Escherichia coli*, pigs, lagoon manure

**INTRODUCTION**

Horizontal transfer mechanism of resistance genes in bacteria plays a primary role in the spread of antimicrobial resistance [29]. The frequency of spread of genetic determinants increases substantially when they are parts of mobile gene cassettes. Gene cassettes exist as free circulating molecules and the carried information is transcribed when they are integrated into integrons by specific recombination sites known as 59 kb elements, recognized by specific recombinase (*intI*) in integrons [6, 13]. The *aadA* genes coding the resistance to streptomycin and spectinomycin in enterobacteria are most commonly described as belonging to different gene cassettes [14, 31, 35]. On the other hand, the *sul1* gene which participates in the expression of resistance to sulfonamides, is part of the 3’ SC conservative sequence in class I integrons together with the *gacEA1* gene coding resistance to quaternary ammonium salts [31, 34]. The *sul2* gene was initially detected in small non-conjugative plasmids, but later its presence was detected in a wide range of conjugative plasmids carrying genes of resistance to streptomycin [2, 15, 30]. In Switzerland, the *sul 3* gene was first described in *E. coli* isolates from farm animals, and afterwards its presence in non-classic class I integrons was established [1].

The resistance to sulfonamides is widely prevalent among swine commensal colibacteria which could be interpreted in the context of the risk for transfer of this resistance type to humans via the food [15].

The aim of this study was to describe the sensitivity of commensal porcine *E. coli* isolates from pigs and manure lagoons to 11 antibiotics, and to determine the genotypic resistance profile to sulfonamides and the presence of *aadA1* and *intI* in studied isolates.
Materials and Methods

**Samples collection.** From January 2013 to May 2015, 470 faecal swab samples were collected from 7 pig farms. The distribution of samples according to their animals’ age category was as followed: from suckling pigs – 157, from growing pigs – 167 and from fattening pigs – 146. Additionally, 104 samples were collected from manure lagoons at studied farms.

**Isolation and identification of Escherichia coli.** The primary isolation of bacteria was done on McConkey agar plates (Emapol, Poland), incubated at 37°C over 24 h. For subcultivation of single colonies, TSI (BD, USA) was used, and for biotyping of isolates – indole production test, growth in Simmons’ citrate agar, methyl red and Voges-Proskauer tests. E. coli strains were identified by means of kits for detection of non-fermentative bacteria and enterobacteria (BBL -ENF, USA) and the semi-automated Crystal BBL System.

Antimicrobial susceptibility testing. For determination of phenotype features of E. coli strains with regard to tested chemotherapeutics, the disk diffusion method was applied according to the requirements of CLSI [4]. Muller-Hinton agar (Emapol, Poland) and disks loaded with chemotherapeutics (Emapol, Poland) were used: ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cephalotin (30 μg), ceftazidime (10 μg), cefotaxime (5 μg), gentamicin (10 μg), streptomycin (10 μg), spectinomycin (25 μg), tetracycline (30 μg), ciprofloxacin (5 μg), and sulfamethoxazole (25 μg). In E. coli bacteria, the MIC values were determined in the disk diffusion test with concentrations of streptomycin between 0.01-256 μg/mL and 8-1024 μg/mL for sulfamethoxazole (Sigma-Aldrich). A control in phenotype test, *Escherichia coli* ATCC 25922 was used. The interpretation of sensitivity of isolates to streptomycin and sulfamethoxazole was done using the epidemiological MIC cut-off values (ECOFF) determined by EUCAST (2015).

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, Germany). The DNA extraction kit DNeasy Blood Tissue Kit (Qiagen, Germany) was carried out according to the manufacturer instructions.

Identification of resistance genes. The *aadA1* genes were determined by qPCR (TaqMan probe). Microbial DNA assay (Qiagen, Germany) was used. The reaction thermal profile included: 1X initial activation of PCR at 95°C for 10 min. The second stage included two steps with 40 cycles of denaturation and annealing/elongation, denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 2 min. Amplification reaction took place in Stratagene Mx3000P.

The positive DNA control was interpreted at values C<sub>90</sub>=34, and positive amplification reaction controls – at C<sub>r</sub>=22±2.

*sul1* and *sul2* genes were determined in conventional PCR. The sequences of used primers were: *sul1*-F 5’ CGT GTG GTA CTT GAA CG 3’, *sul1*-R 5’ GCC GAT CGC GTG AAG TTC CG 3’ and *sul2*-F 5’ CGG CAT GGT CAT AAC CAT CT 3’, *sul2*-R 5’ TGT GCG GAT GAA GTG CTG TC 3’ [20]. The thermal profile of reactions included: 1X 94°C-10 min – initial activation step, followed by 30 cycles including denaturation at 94°C for 30s, annealing at 66°C for 1 min, elongation at 72°C for 30s and one additional extension cycle at 72°C for 10 min. Positive controls were kindly provided by Ms Justina Mazurek, Department of Molecular Biology, Faculty of Biological Sciences, University of Zielona Góra, Zielona Góra, Poland: for the *sul1* gene - *E. coli* 71.3, and for the *sul2* gene – *E. coli* 55.2.

*E. coli* strains carrying the *sul1* and *aadA1* genes were tested for the presence of *intI1* gene [21] and for the variable region in class I integrons, 5’-CS-3’-CS [24]. The primers used for detection of the *intI1* gene were: *intI1*-F 5’ CAG TGG ACA TAA GCC TGT TC 3’, *intI1*-R 5’ CCC GAG GCA TAG ACT GTA 3’. The thermal profile of the PCR reaction consisted of: 1X 94°C - 10 min initial activation step, 30 cycles including denaturation at 94°C for 30s, annealing at 59°C for 30 s, elongation at 72°C for 30 s and one additional extension cycle at 72°C for 5 min. The primers used for detection of the variable region of class I integrons were: 5’-CS-GGC ATC CAA GCA GCA AG 3’ - CS- AAG CAG ACT TGA CCT GA. Thermocycler regime included initial denaturation at 95°C for 12 min, 35 cycles including denaturation (94°C, 1 min), annealing (55°C, 1 min), extension at 72°C for 5 min, and final extension at 72°C for 10 min. Positive controls were also provided by Ms Justina Mazurek, Department of Molecular Biology, Faculty of Biological Sciences, University of Zielona Góra, Zielona Góra, Poland – *intI1*: *E. coli* 36.2. PCR reactions were performed in gradient thermocycler peqStar (Peqlab, Germany). Amplified products were visualized through horizontal electrophoresis with 1.5% universal agarose gel (Peqlab, Germany). Ten μl of each PCR product were pipetted in the agarose gel, previously loaded with GelPilot DNA loading Dye, 5X (Qiagen, Germany). The gel was stained with 10 μg/mL ethidium bromide (Sigma Aldrich), and amplification products were visualised with UV transilluminator. As a marker, 100 bp DNA ladder plus (Qiagen, Germany) was used, and for the protocol for variable region of class I integrons - 1kb DNA ladder plus (Qiagen, Germany) was employed.

Statistical analysis. One-way ANOVA (Graph Pad InStat 3 program) test was used for determining the correlations between resistance genes prevalence and the *E.coli* origin with significance level at p≤ 0.05.
Results

The distribution of *E. coli* isolates among the different age categories of animals and in manure lagoons was as follows: 150 isolates from suckling pigs, 157 – from growing pigs, 142 – from fattening pigs and 91 isolates form manure lagoons.

Table I presents the phenotypic profile of *E. coli* isolates with respect to 11 chemotherapeutics. The highest resistance in commensal *E. coli* isolates (73.3%) was exhibited to tetracycline, followed by streptomycin (69.4%), spectinomycin (63.3%) and sulfamethoxazole (57.7%). With respect to beta-lactams, the isolates resistant to ampicillin were the most numerous (34.6%), followed by those resistant to cephalotin (16.6%) and to amoxicillin/clavulanic acid (2.7%). The distribution of resistant *E. coli* strains isolated from the different age categories and manure lagoons at the farms showed highest percent of strains resistant to aminoglycoside/aminocyclitols (94.9% to streptomycin and 89.1% to spectinomycin) from the group of weaned pigs. In suckling animals, the highest resistance (52.0%) was observed to tetracycline followed by that to streptomycin (26.0%) and to sulfonamides (24.0%). The tendency for high resistant of porcine commensal colibacteria to streptomycin and spectinomycin (87.3%, 81.0%) was also established for isolates from fattening pigs. Most of manure lagoon isolates were resistant to tetracycline (74.7%), and then came the resistance to streptomycin (69.2%) and to sulfonamides (64.8%). The determined MIC_{90} values of isolates were 8μg/mL for streptomycin and 128μg/mL – for sulfamethoxazole.

Table II lists the commonest patterns of resistance in commensal *E. coli* isolated form pigs. Among polyresistant strains, most numerous (27.8%) were isolates resistant to four chemotherapeutics: streptomycin, spectinomycin, tetracycline and sulfamethoxazole. The next most frequent pattern (10.9%) was that of bacteria resistant to beta-lactams ampicillin and cephalotin. Resistance to 5 as well as to 6 drugs was observed in 0.4% of isolates. The respective patterns included resistance to beta-lactams, aminoglycoside/aminocyclitols, tetracycline and ciprofloxacin.

Table III presents the results about the prevalence of the *aadA1*, *sul1*, *sul2*, *intI* genes in porcine *E. coli* isolates. The *aadA1* gene was encountered in 54.4% of commensal colibacteria with high proportion of isolates from growing (81.0%) and fattening (67.3%) pigs. The genetic profile of resistance to sulfonamides showed the presence of the *sul1* gene in 20.3% of strains and the *sul2* gene – in 21.2%; the highest occurrence of the *sul2* gene was detected in isolates from growing pigs (31.8%). The combination of *sul1* and *sul2* was found out in 10.3% of strains, and the simultaneous presence of *sul1*, *sul2* and *aadA1* – in 7.4%. The *intI* gene was detected in 9.8% of tested isolates with most strains originating from the fattening group and from manure lagoons (14.3%). Variable regions with size 1 kb in class I integrons was found in 5.3% of isolates. Statistical significant level at p≤0.05 was determined about the prevalence of *sul1*, *sul2*, *intI* genes between *E. coli* isolates from suckled pigs, weaned pigs and finishers.

Fig. 1, 2 and 3 illustrate the results about the amplification products for *sul1*, *sul2* and *intI* genes of resistant strains from the electrophoresis.

![Figure 1: Electrophoretic separation of products of amplification of a 433 bp fragment of *sul1* gene: Lines 1, 2, 4, 5 9, 13, positive for *sul1* gene; line 18 Positive control; line 19 Negative control; line 20-M- 100 bp DNA ladder](image)

### Antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Suckling pigs (n=150)</th>
<th>Weaned pigs (n=157)</th>
<th>Finisher pigs (n=142)</th>
<th>Manure lagoon (n=91)</th>
<th>Total in swine (n=540)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>31 (20.6)</td>
<td>102 (65)</td>
<td>27 (19.0)</td>
<td>27 (29.6)</td>
<td>187 (34.6)</td>
</tr>
<tr>
<td>Cephalotin</td>
<td>2 (1.3)</td>
<td>5 (3.2)</td>
<td>7 (4.9)</td>
<td>1 (1.1)</td>
<td>15 (2.7)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>6 (4.0)</td>
<td>48 (31)</td>
<td>25 (17.6)</td>
<td>11 (12.1)</td>
<td>90 (16.6)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4 (2.7)</td>
<td>36 (23)</td>
<td>17 (12)</td>
<td>7 (7.7)</td>
<td>64 (11.8)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>39 (26)</td>
<td>149 (94.9)</td>
<td>124 (87.3)</td>
<td>63 (69.2)</td>
<td>375 (69.4)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>32 (21.3)</td>
<td>140 (89.1)</td>
<td>115 (81)</td>
<td>55 (60.4)</td>
<td>342 (63.3)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>78 (52)</td>
<td>132 (84.1)</td>
<td>118 (83.1)</td>
<td>68 (74.7)</td>
<td>396 (73.3)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>3 (2)</td>
<td>11 (7.0)</td>
<td>8 (5.6)</td>
<td>6 (6.6)</td>
<td>28 (5.2)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>36 (24)</td>
<td>118 (75.1)</td>
<td>99 (69.7)</td>
<td>59 (64.8)</td>
<td>312 (57.7)</td>
</tr>
</tbody>
</table>

| Table 1: Prevalence of resistance to 11 tested antimicrobials among faecal *E. coli* from pigs on 7 farrow-to-finish farms |
RESISTANCE AMONG COMMENSAL PORCINE E. COLI ISOLATES

Table II: Patterns of resistance to 11 antimicrobials among E. coli from 7 farrow-to-finish farms (n=540)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Occurrence (%)</th>
<th>95%CL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suckling pigs</td>
<td>Weaned pigs</td>
</tr>
<tr>
<td>Resistance to streptomycin (%)</td>
<td>39(26)</td>
<td>149(94.9)</td>
</tr>
<tr>
<td>Resistance to spectinomycin (%)</td>
<td>32 (21.3)</td>
<td>140 (89.1)</td>
</tr>
<tr>
<td>Resistance to sulfamethoxazole (%)</td>
<td>36(24)</td>
<td>118(75.1)</td>
</tr>
<tr>
<td>aadA1</td>
<td>17 (11.3)</td>
<td>127 (81.0)</td>
</tr>
<tr>
<td>sul1</td>
<td>4 (2.6)</td>
<td>40 (25.4)*</td>
</tr>
<tr>
<td>sul1 + aadA1</td>
<td>1 (0.6)</td>
<td>7 (4.4)</td>
</tr>
<tr>
<td>intI</td>
<td>2 (1.3)</td>
<td>17 (10.8)*</td>
</tr>
<tr>
<td>5'-CS variable region -1kb</td>
<td>-</td>
<td>8 (5.1)</td>
</tr>
<tr>
<td>sul2</td>
<td>7 (4.6)</td>
<td>50 (31.8)*</td>
</tr>
<tr>
<td>sul1 + sul2</td>
<td>7 (4.6)</td>
<td>18 (11.4)</td>
</tr>
<tr>
<td>sul1 + sul2 + aadA1</td>
<td>-</td>
<td>12 (7.6)</td>
</tr>
</tbody>
</table>

Table III: Occurrence of resistance genes aadA1, sul1, sul2, intI, determined among commensal E. coli (n=540) from pigs and lagoon manure

Figure 2: Electrophoretic separation of products of amplification of a 721 bp fragment of sul2 gene: Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 M

Figure 3: Electrophoretic separation of products of amplification of a 160 bp fragment of intI gene: Lines 1, 2, 3, 4, 5, 6, 7, M

Figure 4: Electrophoretic separation of products of amplification of a 721 bp fragment of aadA1 gene: Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 M

Figure 5: Electrophoretic separation of products of amplification of a 160 bp fragment of sul1 gene: Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 M

Figure 6: Electrophoretic separation of products of amplification of a 721 bp fragment of sul2+ sul1 gene: Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 M

Figure 7: Electrophoretic separation of products of amplification of a 160 bp fragment of sul2+ sul1+ sul2+ aadA1 gene: Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 M
Discussion

DEWULF et al. [8] supported the thesis that the frequently encountered resistance to tetracycline, streptomycin and sulfonamides in porcine E. coli isolates could be analysed from the point of view of the selective pressure exerted from the use of these drugs in pig farming from one hand, and with regard to the genetic relationships between genes of resistance from the other. In some European countries (Austria, France, Estonia, the Netherlands) the resistance of commensal E. coli from pigs to streptomycin and sulfonamides is high: >50% [9, 16]. Many researchers also discussed the wide spread of genetic determinants of streptomycin and sulfonamide resistance among porcine colibacterial isolates [11, 19, 20, 33]. SZMOLKA et al. [36] affirmed that the commonest genetic determinants in commensal E. coli isolates from farm animals are aadA1, strA/strB, blaTEM sul1 and sul2, intI, tetA, tetB.

There are controversial opinions with regard to the prevalence of sul1 or sul2 genes in E. coli isolated from farm animals. LANZ et al. [22] reported a prevalence of 8% for the sul2 gene in porcine E. coli isolates. SCHWAIGER et al., VIRVE I. et al., WU et al. [33, 41, 42] reported data about the predominance of sul2 (40%-44%) among commensal E. coli from livestock. GUERRA et al., HAMMERUM et al., MAYNARD et al., SÆNZ et al. [12, 15, 26] demonstrated the wide prevalence of both sul1 and sul2 in E. coli from pigs. MAZUREK et al. [28] provided proofs for the higher prevalence of the sul1 gene in growing pigs during therapy with sulfonamides and trimethoprim.

In Bulgaria, no data have been published on the patterns of resistance to streptomycin and sulfonamides as well as data on the prevalence of genetic determinants among commensal E. coli isolates from livestock species. At the same time, the application of the main groups of chemotherapeutics in pig farming – tetracyclines, aminoglycosides, sulfonamides, beta-lactams, macrolides, creates a selective pressure. Our results about the incidence of polyresistant strains with regard to aminoglycosides, sulfonamides, tetracyclines (27.8%) and their genetic determinants among porcine commensal colibacteria are in line with the thesis of SZMOLKA et al. [36]. As the prevalence of resistance genes sul1 (20.3%) and sul2 (21.2%) was concerned, we did not establish predominance of neither; so, our data are comparable to those of GUERRA et al., HAMMERUM et al., SÆNZ et al. [12, 15, 32].

With regard to the prevalence of class I integrons and the aadA1 gene cassette in particular among porcine E. coli commensals, an attempt for indirect analysis could be made on the basis of strains carrying the indicator genes intI, sul1 and aadA1, as well as on the basis of 1 kb variable regions in class I integrons.

HÖLZEL et al. [17] reported 44.5% prevalence of aadA genes among commensal E. coli from swine. MAZUREC et al. [27] found out aadA1 in 35.0% of streptomycin-resistant E.coli isolates from pigs in association of the widely spread resistance to streptomycin in 88.3% of porcine E. coli strains. SCHWAIGER et al. [33] commented on the 27% occurrence of aadA1 genes in streptomycin-resistant porcine E. coli strains. The data from our study evidencing the predominance of the aadA1 gene (54.4%) in streptomycin-resistant colibacteria were comparable to those of HÖLZEL et al.

In several European countries COCCI et al., KADLEC et al., MAZUREK et al., VAN ESSEN-ZANDBERGEN et al. [5, 18, 28, 40] performed an analysis on the prevalence of the most commonly encountered gene cassettes: aadA1 only and dfrA1-aadA1 in class I integrons in E. coli from livestock.

LAPIERRE et al. [23] established prevalence rate of 57% for the intI gene in commensal E. coli strains from swine and in their opinion, the commonest gene cassette was aadA1. TORRE et al [38] found out that the intI gene was encountered in 33.8% of porcine colibacterial isolates. They presented data for the wider distribution of integrase genes in E. coli from weaned pigs (60%) as well as in fattening pigs (85.8%). Among the isolates from farm environments, the reported prevalence rate was 39.2%. HÖLZEL et al. [17] found out a low prevalence (10.9%) of class I integrons in E. coli isolated from pig manure. The results from the present study also provide proofs for higher percentage of isolates from fattening pigs (14.3%) and from manure lagoons in studied farms.

On the other hand, CARATTOLI et al. [3] analyzed the distribution of aadA genes in streptomycin-resistant E. coli at the background of lack of class I integrons as a possibility for existence of non-integrated gene cassettes in microbial populations. LANZ et al. [22] also discussed the presence of integrons in porcine E. coli which were determined to be sensitive to streptomycin. According to the authors’ opinion, in these cases there was a weak expression of resistance genes within integrons. High levels of gene expression in integrons were observed when gene cassettes were located immediately to 5′ CS [7]. In this study, integrase gene was present in 18.1% of isolates, and we also confirmed the presence of variable region in class I integrons 1 kb of size in 9.8% of isolates possessing the aadA1 gene.

This study reports data about the prevalence of several genetic determinants (sul1, aadA1, intI) related to the presence of class I integrons in commensal E. coli isolates from pigs. The established resistance of more than 50% to streptomycin and sulfonamides was associated in most cases with the aadA1 gene (54.4%), followed by sul2 (21.2%). The intI determinant was found in 18.1% of E. coli strains carrying aadA1.
References


