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Research article

INVESTIGATIONS ON THE RESISTANCE OF PORCINE COMMENSAL *E. COLI* ISOLATES TO BETA LACTAMS

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The aim of this study was to describe the prevalence of antibiotic resistance to beta-lactams and to evaluate two resistance genes *bla* $_{TEM}$ and *bla* $_{OXA-1}$ in *Escherichia coli* isolates from faeces on six swine farms in the Republic of Bulgaria. A total of 186 *E. coli* isolates from 192 faecal swabs were tested by the disk diffusion method to determine resistance patterns to 11 antimicrobial agents. Resistance to beta-lactams was determined by disk diffusion method, E-test, micro-broth dilution method and PCR. About 40.3% of the *E. coli* isolates from swine were resistant to ampicillin. The highest resistance was observed in *E.coli* isolates from weaned pigs to ampicillin – 60.0% and to cephalotin – 45.5 %. The *E. coli* isolates resistant to beta-lactams were examined for the presence of *bla* $_{TEM}$ and *bla* $_{OXA-1}$ genes. The most common *bla* gene identified was *bla* $_{TEM}$, which was found in 92.0% of swine isolates.

Key words: commensal *Escherichia coli*, ESBL, pigs, resistance to beta-lactams

INTRODUCTION

Enzymes hydrolysing beta-lactam antibiotics determine one of the commonest mechanisms of resistance among clinically relevant *Enterobacteriaceae*. Due to the fact that penicillins, cephalosporins, and carbapenems are preferred therapeutic options in the treatment of various infectious diseases in humans, the presence and structure of beta-lactamases are essential for the selection of these chemotherapeutics. Extended spectrum beta-lactamases (ESBL) are hydrolases acting on penicillins, broad-spectrum cephalosporins and monobactams, originating from TEM and SHV-enzymes. The antibacterial therapy in cases where ESBL-producing strains are involved is often impeded further by the presence of co-transfer with participation of plasmids, genes of resistance mediating resistance to aminoglycosides and fluoroquinolones. A horizontal gene transfer of resistance from commensal *E. coli* organisms, possessing ESBL genes to microbial pathogens such as *Klebsiella* spp. is possible [1]. Unfortunately, the efficacy carbapenems which are drugs of choice for treatment of infections caused by ESBL

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producing strains, is also partially compromised due to the spread of resistance in some bacterial species, *Stenotrophomonas* spp. and *Pseudomonas s*pp. TEM-1 that determines the resistance to ampicillin, penicillin and first-generation cephalosporins is involved in 90% of cases of ampicillin-resistant *E. coli* infections [2]. During the last 15 years, the prevalence of ESBL as CTX-M-1, CTX-M-2, CTX-M-14 in Europe, mainly among domestic poultry, was reported by several researchers [3-6]. From the point of view of the risk associated to the selective pressure from use of chemotherapeutics in difference groups of livestock species, it could be pointed out that aminopenicillins are frequently used in different domestic animals and poultry farming in particular. Data of EMEA (1999) demonstrate a relatively high rate of utilisation of penicillins in veterinary medicine in European Union (EC) countries, coming third (9%) after tetracyclines and macrolides. Third- and fourth generation cephalosporins are the second group of choice for treatment of cattle, applied in the therapy of infections as metritis, hoof diseases, mastitis, respiratory infections in ruminants, swine and horses, and skin infections, acute peritonitis, osteomyelitis, and respiratory infections in dogs and cats [7].

In Bulgaria during the latest ten years monitoring of antimicrobial resistance in isolates from farm animals concerns only zoonotic bacterial species like *Salmonella spp.* according the recommendation of European Committee.

MATERIAL AND METHODS

Farms and antibiotic use policy:

Farm – I

Total number of sows – 1100

Antibiotic use policy: Wide use of colistin sulfate for metaphylaxis of post weaning enteritis, 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 was free of dysentery and colonic spirochaetosis, thus did not require the application of tiamulins and tetracyclines.

Farm – II

Total number of sows – 1180

Antibiotic use policy: Wide use of colistin sulfate for metaphylaxis of post weaning enteritis, 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

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

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.

Farm – V

Total number of sows – 410

Antibiotic use policy: Wide use of ceftiofur for metaphylaxis of streptococcal infections in suckling and growing pigs. Wide use of amoxicillin for therapy of erysipelas.

Farm – VI

Total number of sows – 650

Antibiotic use policy: Limited use of antibiotics and a quality biosecurity program. Permanent use of colistin for metaphylaxis of EHEC and ETEC in weaned and growing pigs and also use of gentamicin and enrofloxacin for therapy of enteritis.

With regard to the antibiotic policy related to the use of beta-lactam chemotherapeutics on the six farms, it should be noted that only in three of them, due to frequent outbreaks of erysipelas and problems with *Streptococcus suis* infections in suckling and weaned pigs, beta-lactams, amoxicillin and ceftiofur are more commonly employed.

Sample collection

Between January and May 2014, 192 faecal swab samples were collected from different age groups of pigs (suckling, weaned, finisher) 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) on 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). To determine the sensitivity of isolates to beta lactams, ampicillin, cephalotin, cefotaxime and ceftazidime, the synergic amoxicillin/clavulanic acid (20/10 μg) test was used. Antibiograms were controlled with a reference strain *Escherichia coli* ATTC 25922.

The cephalotin MIC were determined with micro-broth dilution test and cation-adjusted Muller-Hinton broth (Emapol, Poland), by preparation of doubling dilutions of cephalotin (Sigma-Aldrich) within 0.01-128 μg/mL. MIC for ampicillin and amoxicillin/clavulanic acid were defined by Etest strips (AB Biodisk, Solna Sweden). MICs were interpreted according to epidemiological criteria (EUCAST, www.eucast.org).

Determination of resistance genes to beta-lactams 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 beta-lactam antibiotics, *bla* $_{TEM}$ and *bla* $_{OXA-1}$ was detected by PCR. The primers sequences for *bla* $_{TEM}$ gene were F-5′ ATGAGTATTCAACATTTCCG3′,R-5′ CCAATGCTTAATCAGTGAGG3′, for *bla* $_{OXA-1}$ F-5′ACACAATACATATCAACTTCGC-3′, R-5′AGTGTGTTTAGAATGGTGATC-3′. [9,10]. PCR assays in 25μl final volume, contained 12.5 μl Taq PCR Master mix (Qiagen) and 3 μl DNA template. The PCR reaction for *bla* $_{TEM}$ consisted of an initial activation step at 94° C for 5 min, followed by 30 cycles of DNA denaturation at 94^0 C for 1 min, primer annealing at 55° C for 1min, and primer extension at 72^0 C for 1min, and final extension for 10 min at 72^0 C. The PCR reaction for *bla* $_{OXA-1}$ included a 5-min denaturation at 96° C, followed by 35 cycles of denaturation 96° C for 1 min, annealing at 61° C for 1 min and extension at 72° C for 2 min and final extension of 72° C for 10 min. 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 (Peqlab, Germany). Gels were stained with ethidium bromide at 10 μg/mL and visualized by UV transillumination. A 100- bp DNA ladder plus (Qiagen) was used as the marker. Positive control strain *E. coli* ATCC 35218 was used. Negative controls were PCR mixtures with the addition of water in place of template DNA.

RESULTS

Number of isolates: The total number of *E. coli* isolates from examined faecal swabs obtained from different age categories at studied farms was 186.

Resistance patterns

Table 1. Prevalence of antibiotic resistance in E. coli strains from pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates (%)				
	Suckling pigs n= 42	Weaned pigs n= 90	Finishers n=54	Total n=186	95% C.L.
Ampicillin	9 (21.4)	54 (60.0)	12 (22.2)	75 (40.3)	33.4÷47.4
Amoxicillin/clavulanic acid	1 (2.3)	3 (3.3)	0	4 (2.1)	0.5÷4.5
Cephalotin	3 (7.1)	41(45.5)	4 (7.4)	48 (25.8)	19.8÷32.3
Ceftazidime	0	0	0	0	
Cefotaxime	0	0	0	0	
Gentamicin	3 (7.1)	23 (25.5)	4 (7.4)	30 (16.1)	11.2÷21.7
Streptomycin	29 (69)	75 (83.3)	40 (74.0)	144 (77.4)	71.0÷83.1
Spectinomycin	19(45.2)	66 (73.3)	35 (64.8)	120 (64.5)	57.5÷71.2
Tetracycline	28(66.6)	79 (87.7)	32 (59.2)	139 (74.7)	68.1÷80.7
Ciprofloxacin	2 (4.7)	7 (7.7)	0	9 (4.8)	2.2÷8.3
Sulfamethoxazole	17 (40.5)	55 (61.1)	25 (46.3)	97(52.1)	44.9÷59.2

Table 1 presents the results on the spread of resistance patterns among commensal *E. coli* strains isolated from the three age categories of pigs to 11 chemotherapeutics, obtained by the disk diffusion test. The highest percentage of resistance was exhibited to ampicillin – 60.0% of isolates from weaned pigs, 22.2% in finishers and 21.4% from neonatal pigs. With respect to cephalotin, the highest resistance (45.5%) was also determined in weaned pigs, whereas suckling pigs and finisher pigs showed significantly lower incidence: 7.1% and 7.4%, respectively. Resistance to the combination amoxicillin/clavulanic acid was found out in 4 strains (2.1%), originating from one of the 6 farms. In the disk diffusion test, the behaviour of 2 isolates from two different farms to third-generation cephalosporins was characterised with inhibition zones of 20 mm to ceftazidime and 22 mm to cefotaxime. The results from the synergic test with amoxicillin/clavulanic acid however were negative.

Polyresistance patterns in the studied *E. coli* strains showed that the resistance to ampicillin and cephalotin was most frequently seen together with resistance to tetracycline, streptomycin/spectinomycin and sulfonamides (37.6%).

Table 2. Distribution of MICs among commensal *E. coli* (n=186) isolated from pigs

Antibiotic	Cumulative (%) MIC in μg/mL													
	≤ 0.015	0.03	006	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256
Ampicillin						4.8	30.1	45.7	56.0	59.7	82.8	97.8	100		
Amoxicillin/ clavulanic acid							24.2	58.1	85.5	97.8	100				
Cephalotin							2.7	16.6	25.8	34.4	74.2	97.3	100		

Table 2 presents the cumulative percentages of MICs determining the resistance among commensal *E. coli* isolates to beta-lactams. In the studied strains, MIC_{90} was 16μg/mL against ampicillin and cephalotin, and 4 μg/mL to amoxicillin/clavulanic acid.

Occurrence of resistance determinants

Table 3 presents data about the prevalence of resistance genes in *E. coli* and corresponding resistance phenotypes to beta-lactam antimicrobial agents. Among resistant isolates recovered from the different age categories, *bla* $_{TEM}$ was established in those possessing a phenotype of resistance only to ampicillin (73), to ampicillin and cephalotin (44), ampicillin and amoxicillin/clavulanic acid (4). None of strains resistant to beta-lactams have shown *bla* $_{OXA-1}$.

Table 3. Occurrence of resistance genes determined among commensal *E. coli* (n= 186) from pigs

Group of animals	Phenotype of resistance to beta-lactams		Genotype of resistance to beta-lactams	
			bla_{TEM}	$bla_{OXA-1group}$
Suckling pigs n= 42	A	9	bla_{TEM}	-
	A Cf	3	bla_{TEM}	-
	A AMC	1	bla_{TEM}	-
Weaned pigs n= 90	A	54	bla_{TEM}	-
	A Cf	41	bla_{TEM}	-
	A AMC	3	bla_{TEM}	-
Finisher pigs n= 54	A	12	bla_{TEM}	-
	A Cf	4	bla_{TEM}	-

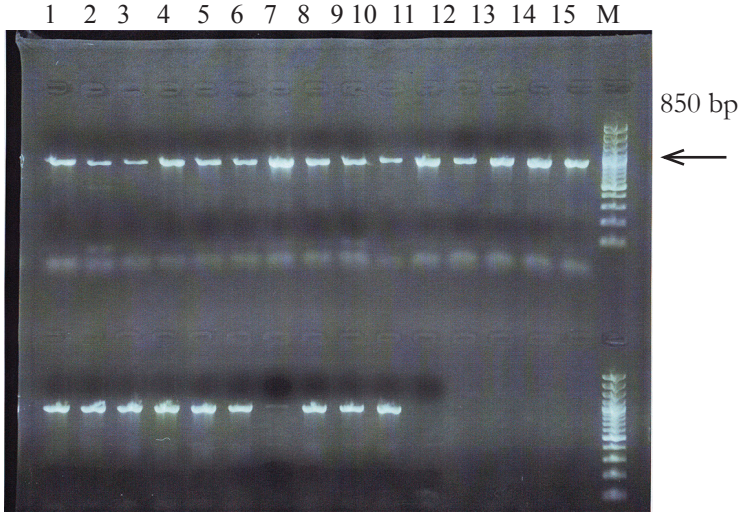

Figure1. Electrophoretic separation of products of amplification of a 850 bp fragment of *bla* $_{TEM}$ gene: Lanes 1 to 24, lane 25-Positive control, lane 26- Negative control, M-100bpDNA ladder.

Fig. 1 depicts electrophoretic patterns of amplification products after amplification of a 850 bp fragment from bla TEM.

DISCUSSION

In the present study, no *E. coli* isolates ESBL producers resistant to cefotaxime and ceftazidime were found, the level of resistance to aminopenicillins (40.3%) and first-generation cephalosporins (25.8%) were high, as well as polyresistant strains (37.6%).

The EFSA report (2011) outlines the particular significance of resistance in indicator *E. coli* from domestic animals to third- and fourth generation cephalosporins and to fluoroquinolones used in medical practice. EFSA stated that reports about the spread of ESBL producing (TEM, SHV, CTX-M) *Enterobacteriaceae* isolated from livestock and foodstuffs have significantly increased during the last decade. Using data from DANMAP (2009), the report noted a low prevalence (0.7%-3.3%) of cefotaxime-resistant *E. coli* isolates from poultry, pork and beef meats and a higher occurrence of resistant E. coli (36%) isolated from imported poultry meat. Also, data about the prevalence of ESBL producers among swine (11%) and the presence of the commonest subtype of CTX-M beta-lactamases – CTX-M-1 (66%) is pointed out. During the last years, a number of authors from several European countries reported data about the incidence of ESBL-producing porcine commensal *E. coli* [13, 14, 15, 16, 17, 18, 19]. In some European countries, a high percentage (52%) of resistance to ampicillin in *E. coli* commensals isolated from cattle, poultry and pigs was outlined [20]. In the Netherlands and Sweden, Van Den Bogaard et al. [21] established a high percentage of commensal *E. coli* isolates from swine, resistant to amoxicillin (51%). Brinas et al. [3] also reported high MIC ≥ 256μg/mL in 62 coli strains isolated from domestic animals and foods. The authors supported the resistance to aminopenicillins with detection of the presence of bla_{TEM} in 83% of resistant colibacteria isolates. In France, data from 2003-2004 published by AFSA [22] provide information about low sensitivity to amoxicillin (39.7%.) in porcine *E. coli* commensals. In Denmark the resistance to ampicillin has increased from 11% in 2002 to 32% in 2004 in *E. coli* strains from pigs [23]. Kozak et al. [24] also determined comparable level of resistance to ampicillin (22%) and 4% to amoxicillin/clavulanic acid in commensal colibacteria from swine. Unlike others, Stannarius [25] described a low resistance to ampicillin (8.6%) in weaned pigs and only 5.8% in finisher pigs.

In the context of reviewed information, it should be noted that during the last decade, antibiotic resistance among indicator bacteria from domestic animals has not been monitored in the Republic of Bulgaria. Such data would permit to perform an analysis and evaluation of risk for spread of resistance in pig farms.

Our data on the resistance to ampicillin are similar to that reported by Wasyl [26] – 42.3% resistance to ampicillin in domestic animal *E. coli* strains on the basis of high MICs – 64 μg/mL, detected in most isolates. Also, the data of Mazurek [27] about

a slightly higher resistance to ampicillin in porcine *E. coli* commensals (49.3%) were attributed to the high prevalence only of *bla* $_{TEM.}$ The authors demonstrated a high resistance to cephalotin – 44.2% compared to our results. The high resistance to ampicillin (95.5%) was also determined in S. Infantis isolated from poultry carcasses in Serbia by Rašeta et al. [28]. The data reported by Guerra et al. [29] are identical to ours as the prevalence of *bla* $_{TEM}$ in 92% of resistant *E. coli* commensals in domestic animals was concerned.

Conclusion: A decade ago, monitoring of the spread of resistance to chemotherapeutics among various pathovarieties of *E. coli* from swine (ETEC, EHEC) in the Republic of Bulgaria established that the prevalence of resistance to amoxicillin and ampicillin was 19.1%. Since then, the criteria for evaluation of resistance to aminopenicillins have changed, which also had some effect on the present results. Taking into consideration that fact that in general, the resistance to chemotherapeutics in pathogenic *Enterobacteriaceae* strains is higher than that observed in commensal *E. coli*, the present study provided evidence about an alarming twice as high resistance to aminopenicillins.

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ISPITIVANJE REZISTENCIJE KOMENSALNIH *E. COLI* IZOLATA OD SVINJA NA BETA LAKTAMSKE ANTIBIOTIKE

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Cilj ispitivanja je bio da se utvrdi prevalencija rezistencije na beta-laktamske antibiotike i da se ustanovi prisustvo dva gena rezistencije *bla* $_{TEM}$ i *bla* $_{OXA-1}$ kod *E. coli* izolovane iz fecesa na šest farmi svinja u Republici Bugarskoj. Kod ukupno 185 E. coli izolata iz 192 brisa fecesa ispitana je rezistenciji prema 11 antimikrobnih agenasa primenom disk-difuzije metode. Rezistencija na beta-laktamske antibiotike utvrđivana je primenom disk-difuzione metode, E-testa, mikrodilucione metode i pomoću PCR-a. Oko 40,3% *E. coli* izolata iz svinja bila su rezistentna na ampicilin. Najveća rezistencija kod *E.coli* izolata iz odlučene prasadi je uočena na ampicilin - 60.0 % i cefalotin - 45,5%. Izolati *E. coli* rezistentni na na beta-laktamske antibiotike ispitani su na prisustvo *bla* $_{TEM}$ i *bla* $_{OXA-1}$ gena. Najčešći bla gen koji je otkriven bio je *bla* $_{TEM}$, koji je pronađen u 92,0% izolata od svinja.