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Antimicrobial resistance rates in commensal *Escherichia coli* isolates from healthy pigs in Greek swine farms

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ABSTRACT: Antibiotic resistance undermines the possibility to effectively treat bacterial diseases in humans and animals and it is one of the major global threats for the future. Antimicrobial resistance among commensal *Escherichia coli* (*E. coli*) of swine is important because it may constitute a reservoir of antimicrobial resistance genes that could be transferred to pathogenic bacteria. This study aimed to estimate the prevalence of antimicrobial resistance among faecal *E. coli* from healthy weaning and growing pigs in Greek farms.

From 14 farms that were enrolled to this study, 390 isolates of *E. coli*, 160 from growing pigs and 230 from weaning pigs had been derived. Isolates were tested for susceptibility to 19 antimicrobials belonging to 10 different classes, using disk diffusion method. Extremely high resistance rates were observed for streptomycin, tetracycline, doxycycline, trimethoprim-sulphamethoxazole, and for the penicillins, ampicillin, ticarcillin and piperacillin. All isolates were susceptible in the combination of a penicillin and β -lactamase inhibitors, in aztreonam and extended-spectrum cephalosporins. The vast majority of the isolates (87%) were multi drug resistant (MDR) and the most common MDR patterns showed resistance in three to four antimicrobial classes. Twenty different antibiotic resistance profiles were observed, the most prevalent was chloramphenicol-trimethoprim/sulphamethoxazole-tetracycline-doxycycline-streptomycin-ampicillin-ticarcillin-piperacillin (CHL-SXT-TET-DOX-SMN-AMP-TIC-PIP) accounting 44% of the isolates. In each farm one or two AMR profiles were predominating accounting 64-100%, while the antimicrobial resistance index (ARI) was estimated to 0.39 ranging from 0.13 to 0.48 among the studied farms. To analyse the differences observed between the farms, additional information about the antibiotic consumption and the level of biosecurity in the farms is necessary. These findings indicate that resistance to a broad range of antimicrobials was prevalent among faecal *E. coli* isolates of pigs on studied farms, and that this constitutes a potential reservoir for resistance genes that could spread to gut pathogens.

Keywords: Antibiotic resistance, *E. coli*, commensal flora, pigs

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INTRODUCTION

The major part of their usage is for treatment of diseases, and as such they have become an essential part of the food-animal husbandry (Marshall and Levy, 2011). Antimicrobial resistance (AMR) undermines the possibility to effectively treat bacterial diseases in humans and animals and it is considered as one of the major global threats for the future. The main driver of resistance is the unjustifiable use of antibiotics, which may cause a selection pressure favouring resistant bacteria and their spread in human as well. Resistant bacteria that emerge among food producing animals can spread to humans, along the food production chain (Silbergeld et al., 2008). Susceptibility testing of commensal intestinal *Escherichia coli* (*E. coli*) from healthy animals is commonly used as indicator for the occurrence of resistance in animal populations. European Union has established surveillance programmes since 2014 (ECDC, 2018; Tadesse et al., 2012).

Antimicrobials are used in animal husbandry for therapeutic reasons, for preventive treatment or prophylaxis, for metaphylaxis or control treatment and as growth promoters however, were banned in EU from 2006 according to European Regulation 1831/2003. The pig production is considered to be amongst the sectors with the highest use of antimicrobials in intensive animal husbandry (McEwen and Fedorka-Cray, 2002). Prophylactic use of antimicrobials to prevent infections was a common practice in pig farms, especially in stressful periods that predispose for infectious diseases. Therapeutic treatments are also administered in feed, although producers also treat individual animals.

Commensal *E. coli* are defined as bacteria isolated from healthy animals without known virulence (toxic, adhesive, invasive) attributes playing a role in a specific disease caused by *E. coli*. Commensal strains of *E. coli* as versatile residents of the intestine are also repeatedly challenged by antimicrobial pressures during the lifetime of their host. As a consequence, commensal strains acquire the respective resistance genes, and/or develop resistant mutants in order to survive and maintain microbial homeostasis in the lower intestinal tract. Thus, commensal *E. coli* strains can be regarded as indicators of antimicrobial load on their hosts (Szmolka and Nagy, 2013).

In the present study, we aimed to estimate the prevalence of antimicrobial resistance among commensal *E. coli* from healthy weaning and growing

pigs in Greek farms and to describe the AMR phenotypic profiles.

MATERIALS AND METHODS

Sampling

Field samples were fecal samples collected at the farm with the permission of the owner of the pig herd and the Official Veterinary Surgeon. The participating farms were purposefully selected from farrow-to-finish operations that had at least 50 sows. Selection was performed from the Greek identification and registration system for livestock (OSDE). A key inclusion criterion was the willingness of the farmer to cooperate at the initiation of the survey. A further inclusion criterion consisted in the absence of other livestock animal species (e.g. cattle, poultry) bred by the selected farms, so that interference of resistance selection due to antibiotic use for these animals was excluded. Fourteen farms located in Central Greece and designated with capital letters (A, B, C etc.) were selected and were categorized as small sized farms <400 sows and big sized farms with 400 or more sows. All animals that have been sampled were healthy and showed no sign of disease.

Samples were taken individually by each animal's rectus with the use of sterile swabs. Samples were kept refrigerated in until analysis within one to four days after sampling. A minimum set of information on the date, location and sample source was collected and submitted to the central database designed for managing of the study. Samples were taken from pigs during the weaning period (3-5 weeks of age) and from pigs during the growing period (16-17 weeks of age); one pig per cage was sampled. From the first four farms (A, B, C, D) 35 animals were sampled 20 weaners and 15 growers. From the remaining 10 farms 25 animals were sampled 15 weaners and 10 growers. Sampled pigs were not treated with antimicrobials during the last month.

Isolation and identification

Samples were inoculated in Tryptone Bile X- Glucuronide Agar (Oxoid, CM0945) and incubated aerobically at $44 \pm 0,5$ °C for 24 ± 2 h. Blue or blue green colonies were determined as *E. coli*. One colony from each dish was picked up randomly and tested for positive indole test for further confirmation.

Antimicrobial susceptibility testing

All isolates were tested for susceptibility against

19 antimicrobials belonging to 10 different classes, using disk diffusion method. For the Quality Control *E. coli* ATCC 25922 was used, while the interpretation and the evaluation of the results was performed according the CLSI (M100S-29th Edition/2019) guidelines. An isolate was considered “resistant” if resistance or intermediate resistance was observed for at least one antimicrobial agent tested. The antibiotics(BIO-RAD), tested were: chloramphenicol (CHL, 30 µg), trimethoprim & sulphomeathoxazole (SXT, 1,75/23,75 µg), nalidixic acid (NAL, 30 µg), ciprofloxacin (CIP, 5 µg), piperacillin (PIP, 100 µg), ticarcillin (TIC, 75 µg), ampicillin (AMP, 10 µg), gentamicin (GMN, 10 µg), tobramycin (TM, 10 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), ceftaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg), aztreonam (ATM, 30 µg), amoxicillin-clavulanic acid (AMC, 10/20 µg), ticarcillin-clavulanic acid (TCC, 75/20 µg), doxycycline (DOX, 30 µg), tetracycline (TET, 30 µg) and streptomycin (SMN, 100 µg). These antimicrobials were chosen because they represent a variety of antimicrobial types. To calculate the rate of resistant isolates per 100 we performed the following calculation

$$\% \text{ rate} = \frac{\text{Number of resistant isolates} * 100}{\text{Number of tested isolates}}$$

Resistance was categorized according to %rate as extremely high (%rate>70%), very high (%rate: >50 to 70), high (%rate >20 to 50), moderate (%rate >10 to 20), low (%rate >1 to 10), very low (%rate 0.1 to 1) and rare (%rate <0.1). Multidrug resistance was defined as previously described (Magiorakos et al., 2012).

We quantified the resistance level by means of antimicrobial resistance index (ARI) which is calculated as the number of antimicrobials against which resistance is detected divided by the total number of antimicrobials tested. For these analyses intermediate results were considered resistant (Hinton et al., 1985). The ARI can vary from 0 (0%), when the strain is (fully) susceptible to every tested antimicrobial agent, to 1.00 (100%) when the strain is (pan-) resistant to all tested antimicrobial agent classes (Catry et al., 2005, Catry et al., 2016)

RESULTS

Overall, 390 *E. coli* strains were isolated; 160 strains were isolated from growing pigs and 230 from weaning pigs.

Overall, extremely high rates of resistance were found for streptomycin (100%), doxycycline and tetracycline (94%), trimethoprim-sulphomethoxazole (93%), ampicillin and ticarcillin (89%), and piperacillin (81%), whereas very high resistance rates were found for chloramphenicol (69%). Moderate resistance rates were found to gentamycin (12%) and trimethoprim (12%) and low to nalidixic acid (6%) and ciprofloxacin (6%). Resistance to cephalosporins, aztreonam and combinations of amoxicillin or ticarcillin with clavulanic acid were not detected. Differences were observed in the resistance rates for each antimicrobial among the 14 farms however most of the strains from each farm shared the similar resistant profiles. Table 1 presents the number and percentages of *E. coli* isolates from pigs found resistant to the antibiotics used in this study.

Resistance to at least one or more antibiotics from three different antimicrobial classes (MDR strains) exhibited 340/390 (87%) isolates while 246/390 (63%) exhibited resistance to at least one antimicrobial from four different classes. Detailed characteristics of the resistance per farm are shown in Table 2.

Table 3 summarises the antimicrobial resistance (AMR) patterns among the 390 *E. coli* isolates. Twenty different patterns were identified among the 390 isolates presenting resistance from two to 12 different antimicrobials. The predominant AMR profile was CHL SXT TET DOX SMN AMP TIC PIP, accounting 173 (44%) of the isolates. The second and third most prevalent were the SXT TET DOX SMN AMP TIC PIP and the CHL SXT TET DOX SMN GEN TM AMP TIC PIP accounting 60 (15%) and 40 (10%) of the isolates respectively.

We observed one to five different AMR profiles in each farm. However, there was a predominant profile for each farm that ranged from 32-100% among isolates. Interestingly the two most prevalent profiles in each farm ranged from 64-100%. The antimicrobial resistance index (ARI) was estimated in 0.39 for all the 390 tested isolates ranging from 0.13 to 0.48 (Table 4).

Table 1: Antimicrobial resistance among *E. coli* isolates from 14 pig farms, Greece, 2014-2015 (N=390)

Farm	N	CHL	SXT	TET	DOX	SMN	GEN	TM	AMP	TIC	PIP	AMC	TCC	CPD	CRO	CAZ	CTX	ATM	NAL	CIP
Total		N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %
A	35	35 100%	34 97%	35 100%	35 100%	21 60%	21 60%	35 100%	35 100%	33 100%	33 94%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
B	35	35 100%	35 100%	35 100%	35 100%	0 0%	0 0%	35 100%	35 100%	35 100%	35 100%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
C	35	35 100%	35 100%	35 100%	35 100%	7 20%	7 20%	35 100%	35 100%	33 100%	33 94%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
D	35	33 94%	34 97%	35 100%	35 100%	9 26%	9 26%	35 100%	35 100%	35 100%	35 100%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
E	25	0 0%	24 96%	25 100%	25 100%	0 0%	0 0%	6 24%	6 24%	6 24%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
F	25	25 100%	0 0%	25 100%	25 100%	0 0%	0 0%	25 100%	25 100%	15 60%	15 60%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
G	25	25 100%	25 100%	25 100%	25 100%	5 20%	5 20%	25 100%	25 100%	21 84%	21 84%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	14 56%	14 56%
H	25	25 100%	25 100%	25 100%	25 100%	4 16%	4 16%	25 100%	25 100%	24 96%	24 96%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	6 24%	6 24%
I	25	0 0%	25 100%	2 8%	2 8%	0 0%	0 0%	2 8%	2 8%	2 8%	2 8%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
J	25	25 100%	25 100%	25 100%	25 100%	0 0%	0 0%	25 100%	25 100%	25 100%	25 100%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
K	25	25 100%	25 100%	25 100%	25 100%	0 0%	0 0%	25 100%	25 100%	23 92%	23 92%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	4 16%	4 16%
L	25	2 8%	25 100%	24 96%	24 96%	1 4%	1 4%	23 92%	23 92%	22 88%	22 88%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
M	25	6 24%	23 92%	25 100%	25 100%	0 0%	0 0%	25 100%	25 100%	24 96%	24 96%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
N	25	0 0%	24 96%	25 100%	25 100%	0 0%	0 0%	25 100%	25 100%	23 92%	23 92%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%
Total	390	271 69%	359 92%	366 94%	366 94%	47 12%	47 12%	346 89%	346 89%	315 81%	315 81%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	24 6%	24 6%

CHL: Chloramphenicol, SXT: trimethoprim & sulphamethoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, PIP: piperacillin, TIC: ticarcillin, AMP: ampicillin, GMN: gentamicin, TM: tobramycin, CAZ: ceftazidime, CRO: ceftriaxone, CTX: cefotaxime, CPD: cefpodoxime, ATM: aztreonam, AMC: amoxicillin-clavulanic acid, TCC: ticarcillin-clavulanic acid, DOX: doxycycline, TET: tetracycline and SMN: streptomycin.

Table 2: Resistance to at least one antimicrobial from different classes among *E. coli* isolates from 14 pig farms, Greece, 2014-2015 (N=390)

Farm	Resistance to antibiotic classes												Total
	1 class		2 classes		3 classes		4 classes		5 classes		6 classes		
	N	%	N	%	N	%	N	%	N	%	N	%	N
A	0	0	0	0	0	0	15	43	20	57	0	0	35
B	0	0	0	0	0	0	35	100	0	0	0	0	35
C	0	0	0	0	0	0	28	80	7	20	0	0	35
D	0	0	0	0	3	9	23	66	9	26	0	0	35
E	1	4	18	72	6	24	0	0	0	0	0	0	25
F	0	0	0	0	25	100	0	0	0	0	0	0	25
G	0	0	0	0	0	0	8	32	15	60	2	8	25
H	0	0	0	0	0	0	15	60	10	40	0	0	25
I	21	84	4	16	0	0	0	0	0	0	0	0	25
J	0	0	0	0	0	0	25	100	0	0	0	0	25
K	0	0	0	0	0	0	21	84	4	16	0	0	25
L	0	0	3	12	19	76	3	12	0	0	0	0	25
M	0	0	2	8	17	68	6	24	0	0	0	0	25
N	0	0	1	4	24	96	0	0	0	0	0	0	25
Total	22	6	28	7	94	24	179	46	65	17	2	1	390

Table 3: Antimicrobial resistance profiles among *E. coli* isolates from 14 pig farms, Greece, 2014-2015 (N=390)

Antimicrobial resistance profile	N	%
CHL SXT TET DOX SMN AMP TIC PIP	173	44.36
SXT TET DOX SMN AMP TIC PIP	60	15.38
CHL SXT TET DOX SMN GEN TM AMP TIC PIP	40	10.26
SXT TET DOX SMN	22	5.64
SXT SMN	21	5.38
CHL SXT TET DOX SMN AMP TIC PIP NAL CIP	18	4.62
CHL TET DOX SMN AMP TIC PIP	16	4.10
CHL TET DOX SMN AMP TIC	10	2.56
SXT TET DOX SMN AMP TIC	8	2.05
CHL SXT TET DOX SMN AMP TIC	4	1.03
CHL SXT TET DOX SMN AMP TIC NAL CIP	4	1.03
CHL SXT TET DOX SMN GEN TM AMP TIC	3	0.77
CHL SXT TET DOX SMN GEN TM AMP TIC PIP NAL CIP	2	0.51
SXT SMN AMP TIC PIP	2	0.51
TET DOX SMN AMP TIC PIP	2	0.51
CHL TET DOX SMN GEN TM AMP TIC PIP	1	0.26
SXT SMN AMP TIC	1	0.26
SXT TET DOX SMN GEN TM AMP TIC PIP	1	0.26
TET DOX SMN	1	0.26
TET DOX SMN AMP TIC	1	0.26

*CHL: Chloramphenicol, SXT: trimethoprim & sulphamethoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, PIP: piperacillin, TIC: ticarcillin, AMP: ampicillin, GMN: gentamicin, TM: tobramycin, CAZ: ceftazidime, CRO: ceftriaxone, CTX: cefotaxime, CPD: cefpodoxime, ATM: aztreonam, AMC: amoxicillin-clavulanic acid, TCC: ticarcillin-clavulanic acid, DOX: doxycycline, TET: tetracycline and SMN: streptomycin.

Table 4: Antimicrobial resistance index (ARI) and proportions of most prevalent and the two most prevalent antimicrobial resistance profiles among 390 *E. coli* isolates from 14 pig farms, Greece, 2014-2015

Farm	Isolates tested (N)	ARI	Number of different profiles	Isolates in the most prevalent profile		Isolates in the two most prevalent profiles	
				N	%	N	%
A	35	0.48	5	19	54	32	91
B	35	0.42	1	35	100	35	100
C	35	0.44	3	28	80	32	91
D	35	0.44	4	23	66	32	91
E	25	0.23	3	18	72	24	96
F	25	0.35	2	15	60	25	100
G	25	0.49	5	8	32	16	64
H	25	0.46	4	14	56	20	80
I	25	0.13	3	21	84	23	92
J	25	0.42	1	25	100	25	100
K	25	0.43	3	19	76	23	92
L	25	0.36	5	19	76	21	84
M	25	0.37	4	17	68	23	92
N	25	0.36	3	22	88	24	96

DISCUSSION

E. coli represents an important aerobic organism in the gut of pigs and other vertebrates, living in symbiosis with its host. We observed high rates of resistance to commonly used antimicrobials and high rates of MDR strains. We observed diversity among the predominant resistance profiles and the antimicrobial resistance patterns in each farm, interestingly; one or two AMR profiles were predominating in each of the studied farms.

We observed high rates of resistance to ampicillin, doxycycline, tetracycline, trimethoprim-sulfamethoxazole, amoxicillin and streptomycin and low rates to nalidixic acid and ciprofloxacin; resistance to cephalosporins or aztreonam was not detected. In accordance to our results, of the *E. coli* isolates from healthy grower-finisher pigs in Canada, resistance was most commonly found to tetracycline (66.8%), sulfamethoxazole (46.0%) and streptomycin (33.4%) (Rosengren et al., 2008). In general, analysis of resistance patterns confirms an increased resistance of isolates to older, frequently administered antibiotics such as streptomycin, chloramphenicol, sulfamethoxazole, and tetracycline (Kang et al., 2005; Österberg et al., 2016; Rosengren et al., 2008; Zhao et al., 2005). In Greece Valiakos et al (2016) reported findings similar to ours such as high rates of resistance to ampicillin, amoxicillin and tetracycline but lower to sulfonamides. According to EFSA report for fattening pigs, the highest overall 'microbiological' resistance levels observed in EU were to tetracycline (54.7%),

sulfamethoxazole (44.2%), ampicillin (39.3%) and trimethoprim (35.3%) with resistance to cefotaxime 1.4% and to ceftazidime 1.3%. MDR resistance was lower in EU (38%) compared to our study, however there was considerable variation between reporting countries in the proportion of isolates which were MDR (EFSA., 2018).

We observed diversity among the predominant resistance profiles and the antimicrobial resistance patterns in each farm although the vast majority of the strains from each farm shared the same profile. We believe that this is possibly due to the use of certain antimicrobials in these farms as there is a direct relationship between AMR and antimicrobial use as suggested also from ECDC (ECDC, 2018). Common classes of antibiotics used in pig production vary across countries. Overall, penicillins and tetracyclines class are the most commonly used antibiotic in pigs, (Legakul et al. 2019) and this can partially match with our findings concerning resistance. Antimicrobial usage in food animals contributes to the development and the spread of resistant microorganisms in the environment but there is also a considerable variation in the antimicrobials consumption all across Europe in terms of differences in antimicrobials used in pig farms (Akwar et al., 2008; Carmo et al., 2017; ECDC, 2017; Garcia-Migura et al., 2014; Gibbons et al., 2016). Animal demographic characteristics, farming practices, veterinarians' and farmers' education are factors which contribute to these variations (Carmo et al., 2017) and can explain the variations in AMR

patterns in different farms.

Although antimicrobial growth promoters have been forbidden in the EU since 2006 (Castanon, 2007), antimicrobials can be used, apart from the direct treatment of diseases, in control treatment called metaphylaxis and this can explain the wide use of antimicrobials in animal husbandry. However, antimicrobials are usually administered to all animals of the group or to the herd; consequently group level use of antimicrobials is the most important way of antimicrobial administration (Callens et al., 2012). We suggest that the predominance of one or two AMR profiles can be partially explained due to similarities in used antimicrobials in our study in farms, as Greek farmers use specific antimicrobials in each farm for prophylaxis or metaphylaxis despite the fact that all the farmers did not mention such use of antimicrobials in the farms.

CONCLUSIONS

The observed high level of resistance to tetracy-

clines, sulfamethoxazole, ampicillin and trimethoprim in *E. coli* may reflect extensive usage of these antimicrobials in the studied farms. According to EMA's 9th ESVAC report that documents the sales of veterinary antimicrobial agents in 31 European countries for the year 2017, for Greece among the most commonly used antimicrobials in farm animals include Tetracyclines with 47,7 mg/PCU, Penicillins with 18,6 mg/PCU and Sulfonamides with 8,3 mg/PDU.

Moreover, we observed one or maximum two AMR profiles predominating in each of the studied farms which again indicates slightly different use of antimicrobials among farms. Antimicrobial usage in food animals contributes to the development and the spread of resistant microorganisms in the environment, prudent uses of antimicrobials is crucial for avoiding spreading resistance to the community.

CONFLICTS OF INTEREST

None to declare

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