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Antibiotic Resistance in Indicator Bacteria Isolated from Cattle and Swine in Greece

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Abstract: Antimicrobial resistance is a phenomenon of increasing importance, as demonstrated by the results of different antimicrobial resistance-monitoring programs. The aim of the present study was to assess the antimicrobial resistance in indicator bacteria isolated from cattle and swine in Greece. The resistance of the bacteria was assessed by the determination of Minimum Inhibitory Concentration (MIC) of each antibiotic used in the study using the microdilution method. *E. coli* isolated from cattle and swine showed resistance to at least one antibiotic used in the study at 75.79 and 88.52%, respectively. The resistance of *E. faecalis* and *E. faecium* isolated from cattle was determined at 68.42 and 66.66%, whereas the resistance of the same bacteria isolated from swine was 81.51 and 72.64%, respectively. Resistance of *E. coli,* isolated from both animal species, to tetracycline and streptomycin was the most commonly observed, although considerable resistance to ampicillin was observed. *Enterococcus* sp. isolated from cattle and swine showed a high level of resistance to tetracycline, streptomycin and erythromycin. In both animal species, multi-resistance occurred in more than 10% of isolated strains of *Enterococcus* sp. and *E. coli.* The results of the study indicate a high level of resistance in indicator bacteria from cattle and swine in Greece, suggesting that a veterinary antimicrobial resistance-monitoring program is needed in Greece to monitor bacterial resistance in animals.

Key words: Antibiotic resistance, indicator bacteria, antibacterial resistance, animal species, amplicilin

INTRODUCTION

Modern food animal production depends partially on the use of antibiotics for disease control and treatment. The pressure from the use of antimicrobial drugs provides favorable conditions for selection and persistence of antimicrobial-resistant bacteria capable of causing infections in animals and humans (Witte, 2000; Aarestrup, 2004). Moreover, some of these bacteria are capable of transferring genetic elements to sensitive bacteria, rendering the recipient organisms resistant to antimicrobials they have never encountered (Cohen and Tartasky, 1997).

Taking into account the ability of bacteria to acquire resistance by acquisition of new chromosomal or extra chromosomal DNA from resistant bacteria of the same or even other species and that food animals as well as food of animal origin is traded worldwide, the occurrence of antimicrobial resistance in one country can be considered a problem for all countries.

During the last decade, awareness of the potential problems that could emerge on the human health front from antimicrobial-resistant bacteria among food-producing animals has increased. Hence, many countries have established monitoring programs for determining the occurrence of resistant bacteria in food animals (Aarestrup, 2004).

The veterinary antimicrobial resistance-monitoring programs are based on determining the resistance of animal pathogens, zoonotic bacteria, as well as intestinal commensal bacteria like strains of *Enterococcus* sp. and fecal *E. coli.* Monitoring of resistance to pathogens isolated from clinical specimens can lead to overestimation of occurrence of resistance, since these samples are not representative of the population. Therefore, monitoring of commensal bacteria resistance gives a more representative estimation of the occurrence of resistance in the entire animal population. In addition, if it is implemented on a regular basis and on different animal populations, the comparison of the prevalence

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through time, as well as the trend and evolution of resistance among species, is feasible. Moreover, the resistance of commensal bacteria is considered a good indicator for selection pressure through antibiotic use on each animal's species and for resistance problems to be expected in pathogens (Lukasova and Sustackova, 2003). Although commensal bacteria are not pathogens, their role in disseminating resistance is important because they constitute a reservoir of resistance genes for pathogenic bacteria and through contamination of products of animal origin can reach the intestinal tract of humans, where they can transfer resistant genes to other bacteria (Lukasova and Sustackova, 2003).

The aim of the present study is to determine antibiotic resistance among the indicator bacteria *E. coli* and strains of *Enterococcus* sp. isolated from swine and cattle feces, in order to assess the antibiotic pressure on these species of food animals. Taking into account that in Greece a veterinary antimicrobial resistance-monitoring program has not yet been established and there is no published information about this issue, the results of the present study can contribute to the illustration of the existing situation and demonstrate if there is a need for additional action for controlling the antibiotic resistance in food animals.

MATERIALS AND METHODS

Collection of samples: Fecal samples were collected from healthy swine and cattle randomly selected from slaughterhouses in Central and Northern Greece during 2005. From each selected animal, a portion of rectum with the content was removed and transferred to a laboratory for isolating the indicator bacteria. Double sampling of the same farm was avoided; therefore, it can be assumed the collected samples were representative of the population reared in these areas.

Isolation and identification of indicator bacteria: For the isolation of *E. coli* and *Enterococcus* sp., 0.5 g of feces was diluted to 4.5 mL of phosphate buffer saline (PBS, pH 7.2) (Sigma-Aldrich Co.) so that a suspension 1:10 w/v was created. The suspension was filtered through sterile gauze in a sterile container in order to remove any solid material. Sequentially, 0.1 mL of the filtered suspension was spread on MacConkey and Slanetz-Bartley agar (Biolife Italiana s.r.l) plates for isolating *E. coli* and strains of *Enterococcus* sp., respectively. The MacConkey agar plates were incubated overnight at 37°C, whereas the Slanetz-Bartley agar plates were incubated at 37°C for 48 h.

One lactose-positive colony with the typical morphology for *E. coli* was selected from every MacConkey agar plate and sub-cultured on blood agar (10% bovine blood). After overnight incubation at 37°C, the isolates were tested for tryptophanase and â-glucuronidase production, using a double test tablet (DIATABS™) for â-glucuronidase (PGUA) and indole test (ROSCO Diagnostica A/S). Only strains showing positive reactions in both tests were selected for further antibiotic susceptibility tests.

Different colonies randomly selected from every Slanetz-Bartley agar plate were sub-cultured on bile-aesculin and blood agar (Biolife Italiana s.r.l). Colonies morphologically consistent to enterococci, catalase negative with positive reaction to bile-aesculin agar were selected. The strains *E. faecalis* and *E. faecium* were identified on the basis of the results of biochemical tests for fermentation of arabinose, mannitol, sorbitol, sorbose and lactose. The biochemical tests were selected from the panel of biochemical tests proposed for identifying *Enterococcus* sp. strains (Manero and Blanch, 1999; Day *et al*., 2001).

Susceptibility testing: The susceptibility of *E. coli* was assessed for Ampicillin (AM), Tetracycline (TE), Chloramphenicol (CHL), Gentamycin (GE), Trimethoprim (TRI), Sulfamethoxazole (SUL), Streptomycin (STR), Neomycin (NE), Ceftiofur (CEF), Enrofloxacin (ENR) and Nalidixilic acid (NAL). The susceptibility of *Enterococcus* sp. was determined for ampicillin, tetracycline, chloramphenicol, gentamycin, streptomycin, neomycin, Erythromycine (ER), Vancomycin (VAN) and Virginiamycin (VIRG) (Sigma-Aldrich Co). The antibiotics were supplied as powders and the stock solutions were created by diluting each one with the solvent and diluent recommended by the manufacturer, taking into account the potency of each antibiotic base. The stock solutions were aliquoted in 1000 μL volume and stored at -70°C until use (NCCLS, 2003).

The susceptibility of indicator bacteria were assessed by definition of Minimum Inhibition Concentration (MIC) for each antibiotic used in the study by broth microdilution method performed in 96 round bottom well microplates at a volume of 0.1 mL, as it is described by NCCLS (now named CLSI) (NCCLS, 2003).

Initially, a series of two-fold dilutions were prepared for each antimicrobial agent in the microplate, diluting properly the stock solution in Mueller-Hindon broth with adjusted cations (Difco®). In each microplate well, 50 μL of the antimicrobial solution was added. The concentration of antimicrobial agent in this solution was double

of the final wanted, because after the addition of equal volume (50 μL) of bacterium inoculum suspension, antimicrobial solution would be further diluted (1:2 dilution). In each microplate, two wells were left as controls in which 50 μL of Mueller-Hindon broth was placed instead of antimicrobial solution.

For preparing bacteria inoculants, suspensions for every bacterium were created in Mueller-Hindon broth with a concentration of 1-2×10^8 CFU mL^{-1}. Bacteria concentration in each suspension was determined by visual comparison with 0.5 MacFarland's standard against a white background with contrasting black lines. The inoculums were further diluted 1:100 adding Mueller-Hindon broth, so suspensions with concentration of 10^6 CFU mL^{-1} were prepared. From these inoculums, 50 μL was added to each well in the microplate, including controls and were mixed with the antimicrobial's agent suspension, resulting in a final concentration of 5×10^5 CFU mL^{-1}.

After the addition of bacteria inoculum, the microplates were sealed with a self-adhering plastic film in order to avoid evaporation and incubated aerobically at 35°C for 18 h.

When the incubation was completed, the microplates were removed from incubator and the results were read by placing the microplate on a viewing device with an enlarging mirror. A bench lamp giving indirect light facilitated reading. Bacterial growth was easily detected in the mirror as a pellet at the bottom of the well.

The MIC for each antimicrobial agent was determined as the lowest concentration completely inhibiting visible growth of bacterium tested.

For quality control, the reference strains of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used.

Data analysis: The collected data were analyzed and the proportions were compared by chi-square test using Medcalc version 8.0 for Windows (Schoonjans *et al.*, 1995).

RESULTS

From the collected fecal specimens, 157 and 122 *E. coli* were isolated from cattle and swine, respectively. Additionally, 114 *E. faecalis* and 147 *E. faecium* were isolated from cattle and 119 and 106 from swine, too.

Table 1: Antibiotic resistance of *E. coli* isolated from cattle and swine

		E.coli from cattle n = 119*		*E.coli* from pigs n = 108*	
Antimicrobial agents	Break point mg L^{-1}	Nr	% of resistant	Nr	% of resistant
Ampicillin	>8	30	25.21	15	13,89
Ceftiofur	>2	2	1.68	0	0.00
Chloramphenicol	>16	7	5.88	5	4,63
Enrofloxacin	>0.25	7	5.88	3	2,78
Gentamycin	>8	2	1.68	5	4,63
Nalidixilic acid	>16	10	8.40	6	5,56
Neomycin	>8	4	3.36	2	1,85
Streptomycin	>32	39	32.77	23	21,30
Sulfamethoxazole	>256	33	27.73	49	45,37
Tetracycline	>8	56	47.06	61	56,48
Trimethoprim	>8	14	11.76	38	35,19

*only resistance strains are examined, ** The sum of isolates is greater because multiple resistance is reported

Table 2: Antibiotic resistance of *E. faecalis and E. faecium* isolated from cattle and swine

		Enterococcus faecalis				*Enterococcus faecium*			
		Cattle n = 75*		Pigs n = 97*		Cattle n = 98*		Pigs n = 79*	
Antimicrobial agents	Break point mg L^{-1}	Nr	%resistant	Nr	%resistant	Nr	%resistant	Nr	%resistant
Ampicillin	>8	8	10.66	7	7.21	18	18.36	11	14.28
Chloramphenicol	>16	6	8.00	12	12.37	5	5.1	3	3.89
Erythrocyne	>4	52	63.33	59	60.82	47	47.95	46	59.74
Gentamycin	>512	7	9.33	21	21.64	8	8.16	14	18.18
Neomycin	>1024	5	6.66	13	13.4	2	2.04	4	5.19
Streptomycin	>1024	54	72.00	71	73.19	31	31.63	17	22.07
Tetracycline	>8	70	93.33	91	93.81	60	61.22	68	86.07
Vancomycin	>16	0		0		0		0	
Virginiamycin	>8	-**		-**		2	2.04	5	6.49

*only resistance strains are examined, ** Not applicable for *E.faecalis*, *** The sum of isolates is greater because multiple resistance is reported

The strains of *E. coli* isolated from bovine and swine, were resistant at least to one antibiotic used in the study at 75.79% (119 out of 157) and 88.52% (108 out of 122), respectively. The resistance of *E. coli* isolated from cattle differs significantly from that of *E. coli* isolated from swine ($p<0.05$). *Enterococcus faecalis* and *E. faecium* isolated from cattle were resistant at least to one antibiotic at 68.42 and 66.66%, whereas for the same bacteria isolated from swine, resistance was calculated at 81.51 and 72.64%, respectively. The resistance of indicator bacteria isolated from cattle was smaller than that of bacteria isolated from swine and differs significantly, as chi-square test reveals ($p<0.05$).

The occurrence of resistance in *E. coli*, *E. faecalis* and *E. faecium* isolates from bovine and swine are presented in Table 1 and 2.

Vancomycin-resistant *Enterococcus* sp. was not isolated from cattle and swine in the present study.

From *E. coli* isolated from cattle and swine, 14.28 and 15.74% showed multiple resistances in more than three of the antimicrobial agents used in this study. From *Enterococcus* sp., 10.40% isolated from bovine and 19.88% isolated from swine showed multiple resistances in more than three of the antimicrobial agents used in the study. The multiple resistances observed in *Enterococcus* sp. isolated from swine fecal samples was higher and differs significantly ($p<0.05$) from that of *Enterococcus* sp. isolated from cattle.

DISCUSSION

In the present study, the resistance of indicator bacteria isolated from swine is significantly higher than that of indicator bacteria isolated from cattle.

This finding is in agreement with the results obtained from veterinary antimicrobial resistance-monitoring systems existing in other EU member states (ITAVARM, 2003; SVARM, 2004). The increased resistance of indicator bacteria isolated from swine feces must be attributed to the intensification of swine production worldwide. In general, the use of antibiotics in ruminants is limited to therapeutic use and not used as growth promoters since the oral administration of antibiotics to ruminant can destroy the flora in the rumen. Thus, the observed resistance of cattle isolates is due to the use of antibiotics for mainly treating diseases. On the contrary, on intensive swine farms where the concentration of animals per unit of surface is higher, the use of antibiotics for treating and for prophylaxis of different bacterial diseases, which are more common in swine, is greater. In many cases, antibiotic compounds are added in the food in sub-therapeutic doses for prophylaxis, contributing to a significantly higher resistant bacteria population in this animal species, exerting a potent selective pressure for emerging resistant clones that already pre-existed in the bacteria population (Corpet *et al*., 1989). For this reason, the antibiotics used in swine production are mainly tetracyclines and combinations of sulfonamides with trimethoprim. For this reason, the results of the vast majority of studies reveal that *E. coli* isolated from swine show a high resistance rate to these antimicrobial agents (ITAVARM, 2003; SVARM, 2004). This has been encountered in the present study, too. The significant multi-drug resistance observed in the isolates from swine can also be explained by the extend use of antibiotics in swine farming.

The most common resistance of indicator bacteria tested from cattle and swine in Greece was to tetracyclines. This finding is in agreement with the findings of similar studies conducted in other parts of the world (Teshager *et al*., 2000). A high level of resistance to tetracyclines was also found in pathogens as well as in indicator bacteria isolated from different animal species in some EU member states where monitoring systems of veterinary antimicrobial resistance are functioning (ITAVARM, 2003; SVARM, 2004).

The widespread resistance of bacteria to tetracyclines can be attributed to the extensive and long-term use of this antibiotic for veterinary therapy, prophylaxis and animal growth promotion in many animal species, resulting in the selection of resistant pathogenic and commensal bacteria (Khachatourians, 1998).

According to the results of several studies, the majority of commensal and pathogenic bacteria in the past were susceptible to tetracyclines, but resistance has emerged due to genetic acquisition of *tet* genes, which encode the resistance mechanisms based on efflux pumps and ribosomal protection proteins (Chopra and Roberts, 2001).

A significant portion of indicator bacteria isolated from cattle and swine showed resistance to chloramphenicol. It must be noted that in the present study all the bacteria resistant to chloramphenicol *E. coli* and *Enterococcus* sp. showed multiple resistance to more than three antibiotics tested in the study.

The existing resistance to chloramphenicol, although this compound has not been used for the last 17 years, must be attributed to the use of other antibiotics, even from different groups and different molecular structures. There is evidence that some resistance to an antibiotic may persist long after its use has been banned. One reason for this would be that the gene , which encodes for resistance to that antibiotic could remain present as a result of the use of other antibiotics to which the determinants are genetically linked on the same plasmid or transposon (co-selection) (Phillips *et al*., 2004).

The *Enterococcus* sp. isolates from cattle and swine showed considerable resistance to erythromycin, although this antibiotic is not commonly used as food additive in swine and is mainly used for therapeutic purposes in cattle. The widespread resistance of *Enterococcus* sp. to macrolide antibiotics can be attributed to the widespread use of other antibiotics belonging in the same group, especially tylosin, which is used commonly for treating respiratory diseases in cattle and swine. Tylosin was used until recently in sub-therapeutic doses in swine, as food additive and for prophylaxis from respiratory diseases. This is supported from the findings of studies about the mechanisms of resistance to macrolides, which reveal that when the mechanism of resistance is based on modification of drug target, the single alteration of the 23S rRNA confers broad cross-resistance to macrolide-lincosamine-streptogramin antibiotics (Portillo *et al*., 2000). To this phenomenon of cross-resistance must be attributed the resistance of *E. faecium* observed in the present study to virginiamycin.

The results of the present study reveal that the *Enterococcus* sp. isolated from cattle and swine express a high level of resistance to aminoglycosides. Penicillin, ampicillin and aminoglycosides are the antibiotics most commonly used for therapy in veterinary practice, especially for infections caused by Gram-positive bacteria, resulting in a high level of resistance. The high-level resistance of *Enterococcus* sp. and especially that of *E. faecalis* isolated from food animals to aminoglycosides (streptomycin, gentamycin and neomycin) are often reported (Donabedian *et al*., 2003). Additionally, it was found that *Enterococci* have also intrinsic resistance to the cephalosporins and are developing widespread resistance to penicillin and ampicillin (Jeljaszewicz *et al*., 2000).

It must be pointed out that in the present study vancomycin-resistant *Enterococci* were not found. This must be attributed to the fact that the use of avoparcin, which shows cross-resistance to vancomycin, was banned by the EU 10 years ago (1997) and that this compound was used mainly in poultry, whereas its use in swine was very limited in Greece.

CONCLUSION

The results of the present study reveal that a considerable resistance has been developed in indicator bacteria in cattle and swine through the use of antibiotics in veterinary practice in Greece. This makes the establishment of a veterinary antibiotic resistance-monitoring system urgent in Greece, as systems have been established in other EU member states, so monitoring the prevalence of resistance in indicator bacteria (*E. coli* and *Enterococci*) as well as in the most important animal pathogens and zoonotic bacteria in different animal populations, can be continued on regular basis. This is necessary because information on the occurrence of resistance is needed at local, regional and national levels to guide the policy for the use of antibiotics in veterinary practice and to detect changes in antibiotic resistance-requiring intervention strategies.

Considering the effect antimicrobial resistance has on human health and also its economic impact, measures to preserve these agents and delay the development of resistance are urgently needed. This includes judicious use of antibiotics in veterinary practice and food animal rearing and implementation of control measures to decrease resistance in reservoirs on farms and in the environment.

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