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Original Contribution

IN VITRO STUDY OF ANTIMICROBIAL SENSITIVITY OF *ENTEROCOCCUS ISOLATES* FROM FARM BIRDS

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ABSTRACT

Five hundred and twenty *Enterococcus* isolates from the intestines of chickens were investigated. Their susceptibility to vancomycin and five other antimicrobials was analysed using the disk-fusion technique. We used the agar-screening method to determine high-level resistance to aminoglycoside-aminocyclitol antibiotics. Nitrocefin-disks for beta-lactamases producers were made. The commonest isolate was *Enterococcus faecium.* The highest level of resistance was recorded against gentamicin and streptomycin, while lowest level was against ampicillin. Resistance to vancomycin was not recorded. On the whole, the differences in levels of resistance among the various *Enterococcus spp* were negligible.

Key words: *Enterococcus spp.,* resistance, vancomycin-resistant enterococcus (VRE).

INTRODUCTION

The resistance to glycopeptide antibiotics is related to altered target and especially, the production of a new target type, not influenced by glycopeptides From a clinical point of view, the most important is the resistance of enterococci against glycopeptides. It is known that in both the USA and Western Europe, the majority of nosocomial infections are aetiologically related to hospital enterococcal isolates (1,2).

The first reports about glycopeptide resistance by clinical enterococcal isolates appeared in 1988 and originated from Great Britain and France.

At present, 4 phenotypes of glycopeptide resistance in enterococci are distinguished. This phenotypic expression is determined by several genetic determinants - Van A, Van B, Van C, Van D. The first glycopeptide resistance phenotype is related to high MIC values to vancomycin and teicoplanin. The second phenotype is characterized by comparatively lower MIC values to vancomycin and preserved sensitivity to teicoplanin. In both cases, the transfer of resistance is mediated via conjugative plasmids or transposons. The third glycopeptide resistance phenotype is determined as constitutive type. In *E. gallinarum* isolates it is known as Van C-1, in *E. casseliflavus* – as Van C-2, and in *E. flavescens* – Van C-3. It is characterized by relatively low levels of vancomycin resistance and preserved sensitivity to teicoplanin. The fourth glycopeptide resistance phenotype is inducible and is expressed under the influence of vancomycin but not teicoplanin. It is characterized by average levels of vancomycin resistance – MIC 64 μg/ml (3).

The possibility of transfer of vancomycin-resistant strains via birds and avian products as well as swine to men is important. It was proposed that one of the causes of onset of such a resistance is the use of avoparcin as growth promoter in chickens and swine in the past. (4, 5).

MATERIALS AND METHODS

Samples and analyses

The sensitivity study was done on enterococci isolated from birds found in 7 different farms in Bulgaria: 5 in South Bulgaria (Stara Zagora, Chirpan, Haskovo, Aytos and Yambol) and 2 in North Bulgaria (Razgrad and Zlatia). The study was carried out between February 2001 – July 2002

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During the investigation, 775 samples of intestinal content from fresh chicken carcasses and 680 samples of faeces of broiler chickens were analysed. From these, 520 strains of enterococci were isolated.

Isolation and identification of enterococci

For cultivation of samples, several nutrient media were used – Columbia nalidixic acid agar (CNA agar), trypticase soya agar with 5% sheep RBC (National Centre of Infectious and Parasitic Diseases), bile-esculin azide enterococcus selective agar (Merck), phenylethyl alcohol agar (Merck).

After inoculation of agar media for 48 hours at 35^{o}C, the suspected colonies of pure cultures (Gram-positive cocci, catalase-negative) were investigated. For identification of *Enterococcus* isolates, the classical schedule of R. R. Facklam & D. F. Sahm was used (6).

The PYR test and the growth of strains in a medium with 6, 5% NaCl at 45 ^{o}C were used as points of reference.

For an easier phenotypic differentiation, the strains were divided into 3 groups. This approach was based on some carbolytic tests including utilization of mannitol, sorbitol, sorbose and arginine.

The first group used for identification included strains with positive carbolytic activity against mannitol, sorbitol and sorbose but with no dehydrogenase activity against arginine.

The second group included strains with carbolytic activity against mannitol and dehydrogenase activity against arginine, but negative against sorbose and a variable behaviour to sorbitol.

The third group included strains positive to arginine and negative to carbohydrates specified in the other 2 groups.

During the identification, additional phenotypic tests were used – determination of the tolerance to potassium tellurite, utilization of pyruvate, saccharolytic activity to raffinose and saccharose.

Determination of the behaviour of avian enterococcal strains to antibiotics

Disk diffusion method

Using the disk diffusion method, the sensitivity of all enterococcal isolates was tested against: penicillin (10 IU), ampicillin (10 μg), gentamicin (120 μg), streptomycin (300 μg) and vancomycin (30 μg). For antibiogrammes, Mueller-Hinton II agar (Becton Dickinson) was employed. The incubation of strains was done at 35^{o}C .The interpretation of inhibition zones around the disks was done according to NCCLS, 1997, Performance Standards for Disc Susceptibility Tests [6th ed. Approved Standard M2-A6. National Committee for Clinical Laboratory Standards, Villanova, Pa (7)].

The inhibition zones around the gentamicin (120 μg) and streptomycin (300 μg) disks were interpreted as sensitive at a diameter ≥ 10 mm; intermediate at 7-9 mm and resistant – at ≤ 6mm. The inhibition zones around the disks with 30 μg vancomycin were assessed as follows: sensitive isolates – at a diameter ≥ 17 mm, intermediate – at 15-16 mm and resistant at ≤ 14 mm. The disks with gentamicin and streptomycin were controlled with the reference *Enterococcus faecalis strains* ATCC 29212.

Agar screening test for detection of high level of resistance to aminocyclitol-aminoglycosides and to vancomycin

For this purpose, Brain Heart Infusion Agar (Difco), containing gentamicin (500 μg/ml); streptomycin (2000 μg/ml); vancomycin (6 μg/ml) was used. The medium was inoculated via spotting of inoculums in stationary phase in a McFarland suspension with an optical density up to 0.5. The cultivation was for 24 hours at 35^{o}C. In case of no growth on the agar with streptomycin (2000 μg/ml) the plates were reincubated for another 24 hours. The method was controlled with the reference *Enterococcus faecalis* ATCC 29212 strain (8).

Nitrocefin disk test

The presence of β- lactamase production, related to high resistance levels to penicillin was determined in enterococcal strains with the ready-to-use Becton- Dickenson disk, impregnated with nitrocefin. A loopfull of growth was streaked on disk surface and then placed in a closed Petri dish. The change in colour of the nitrocefin disk was detected in 15 min (8).

RESULTS AND DISCUSSION

Biochemical identification of avian enterococcal isolates

Table 1 presents the results from the identification of isolates on the basis of the performed tests.

The distribution of isolates is as

follows:

- *Enterococcus faecium* - 480 isolates or 87,27%;
- *Enterococcus faecalis* - 22 isolates or 4,0%;
- *Enterococcus durans* - 8 isolates or 1,45%;
- *Enterococcus hirae* - 6 isolates or 1,09%;
- *Enterococcus gallinarum* - 4 isolates or 0, 72%.

Table 1. B*iochemical tests for Enterococcus spp. isolates*

Biochemical tests	Number of isolates	*Positive Number / %*		*Negative Number / %*	
Haemolysis	520	520	100	0	0
Catalase	520	0	0	520	100
Oxidase	520	0	0	520	100
Motility	520	43	8,3	477	91,7
Pigment	520	43	8,3	477	91,7
PYR-test	495	0	0	495	100
Growth to 45°C In medium with 6,5%Na Cl	520	520	100	0	0
Mannitol	520	506	97,3	14	2,7
Sorbitol	520	22	4,2	498	95,8
Sorbose	520	0	0	520	100
Arginine	520	520	100	0	0
Tolerance to kalii telurit	520	22	4,2	498	95,8
Pyruvate utilisation	520	22	4,2	498	95,8
Arabinose	520	484	93,1	36	6,9
Rafinose	520	10	1,9	510	98,1
Sucrose	520	511	98,3	9	1,7
Esculin hydrolysis	520	520	100	0	0

Determination of the sensitivity of enterococcal isolates to antimicrobials using the disk diffusion test

Table 2 presents some data on the resistance of studied strains enterococci to some beta-lactams, aminocyclitol-aminoglycosides and vancomycin.

The data shown on the **Table** indicate that the highest percentage of resistance was exhibited against streptomycin (45,3%), and the lowest – to ampicillin (9,4 %). The percentage of enterococcal isolates resistant to penicillin was 14% and to gentamicin – 27,1%. There was a clear differentiation in favour of the higher resistance to aminoglycoside-aminocyclitols compared to tested beta-lactam antibiotics.

Table 2. **Resistance to penicillin, ampicillin, gentamicin, streptomycin and vancomycin by 520 strains of enterococci, isolated from birds and determined by the disk diffusion method**

Bacterial species	Number of isolates	Number / (%) of		Resistance isolates to		
		P	Amp	S	G	V
E. faecium	**480**	**72 (15,2)**	**48 (10,1)**	**226 (47,2)**	**136 (28,4)**	-
E. faecalis	**22**	**1 (5,0)**	**1 (5,0)**	**9 (41,5)**	**5 (25)**	-
E. hirae	**6**	-	-	-	-	-
E. durans	**8**	-	-	-	-	-
E. gallinarum	**4**	-	-	**1 (5.0)**	-	-
Total	**520**	**73 (14)**	**49 (9,4)**	**236 (45,3)**	**141 (27,1)**	-

Several differences with regard to the susceptibility of the various enterococcal species were established. Thus, out of the 480 tested *E. faecium* strains, the resistance to penicillin was exhibited by 15,2%, that was close to the average values for all isolates tested. Simultaneously, for *E. faecalis* strains, this percentage was hardly 5% (the difference was significant at $p \geq 0,05$). Similar differences between both species were observed in the susceptibility to ampicillin – 10,1% resistant *E. faecium* and twice lower

levels of resistance for *E. faecalis* (5%).

Against streptomycin and gentamicin, the observed differences for those two enterococcal species were small and insignificant – 47,2% and 41,5% for streptomycin and 28,4% and 25,0% for gentamicin, in *E. faecium* and *E. faecalis* respectively ($p \leq 0,05$).

Those data showed high levels of resistance by both enterococcal species to aminoglycoside-aminocyclitols, with higher percentages of resistance in *E. faecium* isolates –47,2% to streptomycin and 28,4% to gentamicin. For *E. faecalis* the respective percentages were 41,5% and 25%. It is known that the acquired resistance to aminoglycosides and especially to gentamicin is among the most characteristic features of glycopeptide-resistant enterococci.

The performed comparative study with the agar screening method for testing the sensitivity of enterococcal strains to gentamicin, streptomycin and vancomycin confirmed the results obtained using the disk-diffusion test. **Table 3** presents data for intermediate sensitivity to vancomycin using both methods (disk diffusion and agar screen tests). The comparative study using the agar screen method for testing the sensitivity of enterococcal strains to gentamicin, streptomycin and vancomycin confirmed the data obtained using the disk diffusion method. It could be seen that only one strain, identified as *E. faecium* showed an intermediate susceptibility to vancomycin. Around the disk loaded with 30μg vancomycin, an inhibition zone of 16 mm was observed. This intermediate susceptibility was also confirmed in the agar screening test.

Table 3. **Intermediate sensitivity to vancomycin in 520 strains of enterococci, isolated from birds, determined by the disk diffusion and agar-screening methods**

Bacterial species	Number of isolates	Number (%) of intermediate isolates	Number (%) of resistance isolates
		Disc-diffusion method	Screening method
E.faecium	**480**	**1 (0,2)**	**1 (0,2)**
E.faecalis	**22**	**-**	**-**
E.hirae	**6**	**-**	**-**
E.durans	**8**	**-**	**-**
E.gallinarum	**4**	**-**	**-**
Total	**520**	**1 (0,1)**	**1 (0,1)**

DISCUSSION

During the second half of the 1990s, European investigators commented on the relationship between the use of the glycopeptide antibiotic avoparcin as growth promoter and the increasing incidence of isolation of vancomycin-resistant enterococci in their works (1, 2, 4, 5, 9, 10, 11, 12).

In Bulgaria, avoparcin was applied for a short time in the beginning of the 1990s in the broiler and pig-breeding industries.

A study of Jonson et al. (1990) showed that in chickens, the presence of VRE was statistically significantly higher ($p \leq 0,05$) than in adult hens, the difference being considerable as early as the fifth week of life.(1) It is known that the predominating enterococcal flora in chickens under the age of 1 week was represented by *E. faecalis* and *E. faecium*. After the second week, the species diversity changed and *E. faecium, E. hirae* and *E. durans* began to prevail. Later, *E. cecorum* was also isolated. Avoparcin is used as growth promoter in growing chickens. Hen's forage is supplemented with bacitracin, virginiamycin and bambermycin as nutritive antibiotics. As mentioned already, chickens and broilers in particular, are the main sources of VRE among animal populations. The relationship between the use of avoparcin and the isolation of VRE from growing chickens was responsible for the prohibition of this growth promoter in Denmark in 1995. Three years later, the use of virginiamycin and other glycopeptides as nutritive agents was also banned.

Vancomycin-resistant enterococci are widespread in the USA, especially in hospitals (2). Data obtained in 1993 from the US Center for Disease Control and Prevention showed them as the commonest causes of nosocomial infections or infections of large communities (13). Unlike the European countries, the isolation of VRE from animals in America was a relatively rare event almost to the end of the last century (14, 15).

The problem became important for the USA only after their isolation from chickens and turkeys (16) although neither in the USA nor in Canada, was avoparcin officially registered and allowed for use. From an

epidemiological point of view, Fred C. Tenover stated that one of the possibilities for transfer of resistance (apart the use of avoparcin) occurred along the pathway of the food chain, i.e. through the consumption of animal foodstuffs contaminated with resistant enterococcal strains (17). An indirect proof of this assumption is the presence of VRE in faeces of healthy volunteers from Europe, of healthy animals and environmental sources. This is an evidence of the fact that the presence of these microorganisms as part of the normal microflora probably permitted their involvement in the food chain and, therefore, could be one of the ways for the spread of resistant branches (18, 19, 20, 21, 22, 23).

Probably, our results about the lack of vancomycin-resistant enterococcal avian isolates could be determined by the short-time use of avoparcin in Bulgaria. The importance of the duration of the selection process is indicated by the results of Butaye et al. (1999) declaring that the use of this glycopeptide antibiotic could result in the appearance of glycopeptide-resistant enterococci after a long period post application, i.e. the process was time-consuming. (24, 25, 26).

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