Epidemiology of \textit{vanA} gene carrier enterococci: Molecular characterisation, antibiotic sensitivity and phylogenetic relationship of Hungarian isolates

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INTRODUCTION

Enterococci very frequently cause serious nosocomial infections, urinary tract infections and often colonize catheters and other implants. Glycopeptides are the last resort in the treatment of multi-resistant Gram-positive bacteria and due to the rapid spread of resistance to these antibiotics, these infections are now very difficult to treat (1). The glycopeptides include vancomycin and teicoplanin used in human therapy, as well as avoparcin, a growth promoter in animal husbandry. The emergence of the first vancomycin-resistant Enterococci (VRE) in clinical isolates in Europe was published in 1988 (2, 3). Today, 19 years later, glycopeptide resistance (GR) in enterococci is widespread all over the world. The increasing use of other classes of antibiotics, such as aminoglycosides, cephalosporins, and quinolones for infections caused by Gram-negative bacteria and glycopeptides for infections caused by other Gram-positives (staphylococci and Clostridium difficile) has contributed to the emergence of GRE (4). The resistance genes from vancomycin-resistant enterococci (VRE) can be transferred to other bacteria among others to methicillin-resistant Staphylococcus aureus (CDC, 2002 MMWR 51, 565–567).

Correlation was also demonstrated between avoparcin usage and glycopeptide resistance of the human isolates (5, 6). Therefore, the Commission of the European Union (EU) has banned the use of the food additive avoparcin across Europe from April 1997, and one year later the ban was extended also to Hungary.

Glycopeptide resistance in enterococci is encoded by van genes. VanA and vanB genes are the two most significant representatives. VanA confers high-level inducible resistance to both vancomycin and teicoplanin, and it is most often found in E. faecium, but is also present in E. faecalis and occasionally in other species (7). This gene was present in the first resistant clinical isolates. VanB gene was discovered one year later (8) and this phenotype is characterized by only moderate to low-level resistance to vancomycin, and sensitivity to teicoplanin (7). Later also other van genes were described, for example vanC, vanD, vanE, vanG (9, 10, 11, 12).

Those strains of animal origin, which get into the human gut by polluted raw meat or foodstuffs, may present difficulties in humans. Monitoring antibiotic resistance and relationship of enterococci isolated from slaughtered healthy animals is a useful tool to get information about the human risks of these strains.
AIMS

The purpose of this work was to perform an epidemiological study of Hungarian vancomycin-resistant enterococci (VRE), isolated at the Central Veterinary Institute from healthy broiler chicken intestine after ban of the use of avoparcin, in a 4 year period between 2001-2004, within the confines of Hungarian Antibiotic Resistance Monitoring System. We included also the characterisation of the human VRE strains isolated in Hungary. First we wanted to determine the antibiotic resistance pattern of the enterococcal strains from healthy poultry in that period. We tried to identify the vancomycin-resistant strains at genus and species level by biochemical reactions and by PCR. The van genes are responsible for vancomycin-resistance and we investigated the prevalence of van genotype of glycopeptide-resistant enterococci (GRE) from animal and human sources in Hungary. Because the sources of the animal strains were registered at county level, we examined which Hungarian counties had the highest prevalence of VRE strains. We based the epidemiological examination on a genotyping (PFGE) method; we determined the heterogeneity of animal VRE and human VRE isolates from hospital. With specific software we tried to establish whether only one clone spread all over the country, or rather if different types of strains emerged independently. Theoretical we investigated the effect of the avoparcin prohibition on glycopeptide resistance in enterococci. During the classical and molecular identification we developed a biochemical flowchart and a DNA extraction protocol for PFGE examination.

MATERIALS AND METHODS

Bacterial strains

The animal strains investigated in the study were isolated from slaughtered healthy animals within the confines of the Hungarian Antibiotic Resistance Monitoring System.

Glycopeptide-resistant enterococci from poultry were collected from January 2001 to December 2004. Every month since January 2001, the local veterinary authorities of the 19 Hungarian counties have sent the entire caecum of healthy slaughtered animals
to the Central Veterinary Institute.

Intestinal contents were diluted 1/10 (w/v) in a phosphate-buffered salt solution and a drop of this suspension was streaked on Enterococcus agar plates and Columbia blood agar supplemented with 0.5 g/L potassium-tellurite. Strains that were able to grow on agar with tellurite and were catalase-negative, amylase-negative and aesculin-positive, were identified as *Enterococcus faecalis* (13, 14). Bacteria from other colonies were identified at genus level by PCR (15, 16, 17). Vancomycin-resistant strains were identified at species level by PCR and the examination of the *van* genes also were done by PCR.

The first VRE strain in Hungary, an *Enterococcus faecalis* originated from the purulent discharge of an ulcer of the toe from a diabetic male patient. Beside the traditional routine methods, identification was carried out by means of the Rapid ID 32 Strep kit (BioMérieux) according to the manufacturer’s instruction. Antibiotic susceptibility testing was performed with an ATB STREP test (18).

The second VRE strain in Hungary originated from the wound in the deep colliquating necrotised tissue of a patient, who suffered by *Neisseria meningitidis* serogroup C infection (19).

**Antimicrobial susceptibility testing**

The minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin were determined by agar dilution test on MHA plates according to the BSAC guidelines (British Society for Antimicrobial Chemotherapy Working Party, 1991), with a Denley multi-point inoculator. After drying the inocula, plates were incubated at 37°C for 18-24 hours. The MIC was defined as the lowest concentration of an antibiotic to inhibit all visible growth. The MIC<sub>50</sub> and MIC<sub>90</sub> (concentration of antibiotic required to inhibit 50% and 90% of bacterial strains, respectively) were determined when appropriate.

**Identification the GRE isolates by PCR**

The DNA template for the PCR was prepared by heating bacterial cells (a loop-full bacteria suspended in 0.1 ml sterile deionized water) to 99 °C for 15 minutes in a Perkin-Elmer thermal cycler. Following centrifugation to remove debris (6000rpm 1
min in Sorvall RMC-14), 2.5µl of supernatant was used as template in the PCR reaction.

**PCR primers for identification**

For genus-specific identification the primers were designed for the 16S rRNA gene sequences (17). For species-specific identification the enterococcal superoxide dismutase (sodA) gene sequences were used (20).

**Detection of glycopeptide resistance genes by PCR**

All isolates exhibiting high-level resistance to vancomycin and variable resistance to teicoplanin were presumed to be of the VanA-type. The predicted phenotypes were confirmed by PCR detection of the glycopeptide resistance genes with the vanA-specific primers (21). To exclude the presence of the other van genes in the strains, they were examined also with vanB, vanC₁ and vanC₂ primers.

All PCR products were electrophoresed in 1.5% agarose gels in TAE buffer, alongside a 100-bp DNA ladder. After electrophoresis, gels were stained in 0.5 mg/L ethidium bromide and visualized on a UV transilluminator.

**Pulsed-field gel electrophoresis**

Pulsed-field gel electrophoresis (PFGE) was applied to assess the genetic relatedness of isolates. PFGE was performed by an internal protocol, using the CHEF-DR II (Bio-Rad, Hungary) system. The plugs were digested by 10U of SmaI at 25°C for 2 hours. Running parameters of the samples was defined by Murchan et al. (22): block-1 switch 5-15s, 6V/cm, 10h and block-2 switch 15-60s, 6V/cm, 13h. The angle was a constant 120° and the ramp factor was linear. Buffer temperature was 14°C. The gels were stained with ethidium bromide (0.5 mg/L) for 15 minutes. Photographs of gels were digitalized by a Kodak DC290-Zoom camera, banding patterns were compared and the dendrogram was set up by both the Diversity Database and Fingerprinting software (Bio-Rad, Hungary), tolerance: 2.5% method: Dice coefficient, cluster method: UPGAMA. The results of the comparisons were obtained in two forms: phylogenetic tree and similarity matrix. The threshold for the isolates belonging to the same clone was defined as 90% similarity under these conditions.
RESULTS AND DISCUSSION

Antibiotic sensitivity of the human \textit{vanA} gene carrier strains in Hungary

The first VRE strain, confirmed by PCR, isolated in Hungary in 1998 had a MIC of vancomycin of $>256$ mg/L and teicoplanin of 96 mg/L. The strain was sensitive to ampicillin, cotrimoxazole and rifampicin. Cefuroxime, erythromycin, lincomycin, vancomycin, teicoplanin, tetracycline and oxacillin were ineffective \textit{in vitro} against the strain.

A strain isolated from the patient suffering from meningococcal meningitis in 2001 was the second \textit{vanA} positive VRE strain (confirmed by PCR) in Hungary. By antibiogram this strain was multi-resistant and was only sensitive to ampicillin and rifampicin. The MICs of vancomycin and teicoplanin were 256 mg/L and 32 mg/L, respectively. PCR amplification with \textit{vanA} primers in both strains revealed the presence of the \textit{vanA} genes.

Antibiotic sensitivity of the \textit{Enterococcus} strains isolated from poultry in Hungary

During the examination period (2001-2004), the antibiotic susceptibilities of a total of 562 isolates were tested. All but 7 broiler chicken isolates from the year 2001, 1 from 2002 and 1 from 2003 were susceptible to ampicillin. All strains were sensitive to gentamicin. Among broiler chicken isolates, 4 in 2001 and 1 in 2002 were intermediate and 8 in 2001, 7 in 2002, 6 in 2003 and 1 in 2004 were resistant to streptomycin. The number of strains with reduced susceptibility to erythromycin was nearly as high as the number of tetracycline-resistant strains and susceptibility was also reduced both in 2003 and in 2004 in strains isolated from poultry. Among the isolates from chickens, tetracycline susceptibility was lower than 50% in every year examined. The number of tetracycline-resistant and intermediate-resistant strains increased in 2003 and 2004. Among the broiler strains, 34 were intermediately and 68 fully resistant to vancomycin in 2001. These numbers diminished to 6 and 2 in 2004, respectively.
**Extraction of DNA for PCR**

Several methods are published in the international literature about the DNA extraction. Some protocols are too expensive, others are very time-consuming. In the beginning a special protocol, developed for *Staphylococcus aureus* was used. Although this procedure was very quick (ready in 45 minutes) and needed only Tris buffer, lysostaphin and proteinase K, it was expensive because of the lysostaphin; so we tried the simplest and cheapest boiling method for DNA extraction. As we obtained the same results by boiling, only this method was used in the further experiments. The extracted DNA contains cell wall, protein and membrane remnants, therefore, is not good as a template for PCR reactions where the product size is longer than 1000 base pairs. The DNA sample is stable for years at -20°C.

**Extraction of DNA for PFGE**

PFGE examination requires a much more purified DNA sample than PCR. Our research group tried several unpublished and published protocols but all were very difficult and time-consuming. All protocols were ready in one week and used special reagents or chemicals. The procedure we have finally chosen was originally developed for *S. aureus* and required only one day to complete. Additionally, this protocol is very simple, fast and used only three buffers, therefore it was used for the extraction of enterococcal DNA. The enzyme responsible for the digesting of the cell wall was lysostaphin. This enzyme could digest most enterococcal strains but in some cases proved to be ineffective. Therefore we modified the protocol by using additional lysozyme for DNA extraction with longer incubation time (three hours instead of one hour). These changes resulted in successful DNA extraction of lysostaphin resistant enterococci. Lysostaphin is a glycylglycine endopeptidase which specifically cleaves the pentaglycine cross bridges found in the peptidoglycan. The lysozyme enzyme functions by attacking peptidoglycans and hydrolyzing the glycosidic bond that connects N-acetyl muramic acid with the N-acetylglucosamine. The cell wall structure of lysostaphin resistant bacteria remains unknown.
Detection of the \textit{van} genes by PCR and identification of the VRE isolates from chicken at genus and species level

Examination of the strains with primers for the \textit{van} genes revealed that all VRE in this study were vancomycin-resistant because of the presence of the \textit{vanA} gene, and no others – including \textit{vanB} – could be detected. These strains were confirmed to be \textit{Enterococcus} by genus specific PCR, and the identification at species level, also performed by PCR, showed that just three species were represented in this cohort: \textit{E. faecium}, \textit{E. durans} and also one \textit{E. mundtii} strain.

Eleven \textit{E. faecium} strains were isolated in 2001 and 9 in 2002. Nearly similar results were obtained with the \textit{E. durans} strains: 13 strains were isolated in 2001 and 11 in 2002. Only one \textit{Enterococcus mundtii} strain was isolated in 2001. We could not find any \textit{E. faecalis} strains among the \textit{vanA} gene carriers. In 2003 and 2004, we did not find any \textit{van} gene carrier strains.

The MICs of vancomycin and teicoplanin of VRE strains isolated from broiler chickens

In 2001, 11 \textit{E. faecium} were identified as VRE and the MICs of vancomycin of all strains (100%) were higher than 256 mg/L. In 2002, only one strain (11%) had an MIC of vancomycin higher than 256 mg/L. Seven strains (78%) had an MIC of 256 mg/L and only one \textit{E. faecium} strain (11%) had an MIC of 64 mg/L.

The distribution of the MICs of teicoplanin of the vancomycin-resistant \textit{E. faecium} strains isolated in 2001 and 2002 was more diverse. In 2001, three strains (27%) had very high MICs of teicoplanin (>256 mg/L), one strain (9%) had 256 mg/L, 4 strains (36%) 128 mg/L, 1 strain (9%) 64 mg/L and 2 strains (18%) 32 mg/L. In 2002, only one strain (11%) had higher than 256 mg/L MIC of teicoplanin. None of the strains had 256, 128 or 64 mg/L MIC of teicoplanin in that year. One strain (11%) had 32 mg/L, 5 strains (55%) had 16 mg/L, and 2 strains (22%) had 8 mg/L.

In 2001, most of the strains had an MIC of teicoplanin higher than 32 mg/L. In 2002 the dominant MIC value of teicoplanin was 16 mg/L.

The MICs of vancomycin of \textit{E. durans} strains was a little different. All of the examined strains from 2001 had an MIC of vancomycin higher than 256 mg/L, except
for one strain (8%) with an MIC of 32 mg/L. Among the strains isolated in 2002, the MIC values varied between 64 mg/L and >256 mg/L. Only one strain (9%) had an MIC of vancomycin >256 mg/L and 2 strains (18%) had 256 mg/L, 3 strains (27%) had 128 mg/L and 5 strains (45%) had 64 mg/L.

The distribution of the teicoplanin MICs among vancomycin-resistant *E. durans* strains was more interesting. In 2001, 5 strains (38%) had higher MICs of teicoplanin than 56 mg/L. One strain (8%) had 256 mg/L, 2 strains (15%) had 128 mg/L, 2 strains (15%) had 64 mg/L and 3 strains (23%) had 16 mg/L. In 2002, the distribution moved towards the lower intervals, only 2 strains (18%) had an MIC of teicoplanin >256 mg/L, none of the strains had 256, 128 or 64 mg/L. One strain (9%) had 32 mg/L and the dominant value with 4 strains (36%) was 8 mg/L MIC of teicoplanin, but also teicoplanin-sensitive strains appeared.

The MIC required to inhibit the growth of 50% or 90% of organisms (MIC$_{50}$ and MIC$_{90}$) of vancomycin of *E. faecium* strains were >256 in 2001 and =256 mg/L in 2002. Both the MIC$_{50}$ and MIC$_{90}$ of vancomycin for *E. durans* strains were >256 mg/L in 2001, while in 2002 only 128 and 256 mg/L, respectively.

**Geographical source of the VREs**

In 2001, two vancomycin-resistant *E. faecium* strains each were isolated from Baranya, Borsod-Abaúj-Zemplén and Pest counties; and one strain each from Győr-Moson-Sopron, Jász-Nagykun-Szolnok and Somogy counties. None from Bács-Kiskun and Komárom-Esztergom counties. In 2002, three *E. faecium* isolates originated from Veszprém, two strains from Fejér and one each from Bács-Kiskun, Győr-Moson-Sopron, Hajdú-Bihar and Komárom-Esztergom counties. We could not isolate VRE strains from Baranya, Borsod-Abaúj-Zemplén, Pest, Jász-Nagykun-Szolnok and Somogy counties.

In 2001, two *E. durans* strains each were isolated from Baranya, Borsod-Abaúj-Zemplén and Pest counties and one each from Győr-Moson-Sopron, Jász-Nagykun-Szolnok and Somogy counties. In 2002, three isolates originated from Veszprém county, two strains from Fejér and one strains from Bács-Kiskun, Győr-Moson-Sopron, Hajdú-Bihar, Jász-Nagykun-Szolnok and Komárom-Esztergom counties. In 2001 and 2002 not a single VRE was isolated from Békés, Csongrád, Nógrád,
Szabolcs-Szatmár-Bereg, Tolna and Zala counties. Vas county is an exception because in 2001 a vancomycin-resistant *E. mundtii* strain was isolated.

**Comparison of the programs used for creating the dendrograms**

At the beginning the Diversity-Database (Bio-Rad) software was used for analysing the PFGE gels. This software requires a manual matching the DNA bands, therefore it is very difficult to set up a precise dendrogram. The selection of the strains by different properties for drawing the relationship is difficult and sometimes impossible. Recently we had access to a fully operating version of the Fingerprinting software from Bio-Rad Hungary Ltd. This is a modern and widely used program with many special functions, and with the help of this we could make many different selections among the VRE strains and could analyze their relationship easily.

The dendrograms of the vancomycin-resistant *E. faecium* and *E. durans* strains were created based on the PFGE patterns.

With this software the dendrograms were created according the species and isolation date. In 2001, 11 *E. faecium* strains were isolated with very high (>256 mg/L) MICs of vancomycin. Two identical strains were found in that year. One strain originated from Borsod-Abaúj-Zemplén and the other one from Pest county. The MICs of vancomycin were the same and the MICs to teicoplanin were 32 and 128 mg/L. Another pair of strains had very closed relationship (95% of similarity) but were not fully identical. The origin of these strains was Jász-Nagykun-Szolnok and Borsod-Abaúj-Zemplén counties. In that year two *E. faecalis* strains each were isolated from Baranya, Pest and Borsod-Abaúj-Zemplén counties. These strains were not identical which demonstrates the polyclonal origin of vancomycin resistant *E. faecium* strains in 2001.

In 2002, nine vancomycin-resistant *E. faecium* strains were isolated. Four strains were identical; these had an MIC of 256 mg/L to vancomycin and 8 or 16 mg/L to teicoplanin. The origin of these strains was Komárom-Esztergom, Győr-Moson-Sopron, Bács-Kiskun, and Fejér counties. These counties, with the exception of Bács-Kiskun, are found in the North-Western region of Hungary and border one another. This identity permits us to declare the probability of monoclonal origin of the vancomycin-resistant *E. faecium* in 2002. One pair of identical strains originated from
Veszprém county, which further supports our hypothesis about the origin of the strains. All counties with the exception of Hajdú-Bihar and Bács-Kiskun are located in the Western part of Hungary. We had only one vancomycin-resistant *E. faecium* strain from Hajdú-Bihar from 2002, however, the similarity of this strain with the others was lower than 35% and its MIC to teicoplanin was very high (>256 mg/L), unlike in the other cases.

In 2001, 13 vancomycin-resistant *E. durans* strains were isolated from different counties of Hungary, and the Eastern region of Hungary was also represented by Szabolcs-Szatmár-Bereg, Békés and Csongrád counties. None of the strains were identical in the examined period. The MICs to vancomycin were >256 mg/L in almost all cases, except one strain that had only 32 mg/L. The MICs to teicoplanin varied from 16 to >256 mg/L. These data strongly suggest the polyclonal origin of these strains.

In 2002, 11 strains were isolated as vancomycin-resistant *E. durans* strains, but none of the strains were identical. Two strains were closely related (>90% similarity), both with an MIC of vancomycin of 64 mg/L. Their MICs to teicoplanin were 8 and 4 mg/L. The origin of these strains was Borsod-Abaúj-Zemplén and Hajdú-Bihar counties, both are located in the Eastern part of Hungary.

If the search criteria were set up entirely based on the high vancomycin MICs (>256 mg/L) and the species or the isolation date were not taken into account, the dendrogram obtained was very interesting. The polyclonal origin of the *E. durans* strains becomes evident, as the origin of these strains was from all regions of Hungary. On the other hand, the monoclonal origin of the vancomycin-resistant *E. faecium* strains is also clear, as five identical strains were found.

**CONCLUSIONS**

We examined the enterococcal isolates of a large cohort of healthy Hungarian broiler chicken and also a few available human cases, collected at different parts of the country, including reliable identification, antibiotic sensitivity testing and genotyping of the isolates, using international guidelines and molecular methods. Because of the uncertainties of the routinely used methods, the identification of the strains was also confirmed by a molecular technique, the PCR amplification of the *ddl* <i>E. faecium</i> and *sod*
gene. Additionally, we have designed a flowchart for the exact identification of the different enterococcal species, based on their biochemical properties.

In this present work the antibiotic susceptibility of enterococci isolated from slaughter animals were investigated within the confines of Hungarian Antibiotic Resistance Monitoring System. It was very important to introduce the antibiotic monitoring system to obtain some information about the level of the antibiotic resistance and to follow the changes year by year.

Among human isolates we also found vancomycin-resistant ones, and by detection of the \textit{vanA} gene by PCR we demonstrated undoubtedly that VRE strains are present not only in the animal, but also in the human population in Hungary. The first Hungarian human VRE was found and characterised by our work group, including confirmation by PCR. After the determination of the antibiotic sensitivity levels, we found contradictory high vancomycin resistance rates among chicken isolates after three years of the prohibition of avoparcin. Additionally, we found high tetracycline and erythromycin resistance. This could probably be explained by the presence of linked resistance found on transposons, and we would like to extend our study to investigate the molecular background of this.

The vancomycin resistance results confirmed the predominance of \textit{vanA} gene in the Hungarian chicken population. Interestingly, there were 2 isolates with \textit{vanA} gene found also in human isolates which is very rare. Recently \textit{vanB} genes were found in human VRE isolates.

Among chicken isolates we found vancomycin-resistant \textit{E. faecium} and \textit{E. durans} strains and only one \textit{E. mundtii} strains. The number of publications of vancomycin-resistant \textit{E. mundtii} is very low, this type of strain is very rare in the environment, therefore the identification kits are not able to identify it at species level. According to our results, now the diagnostic laboratories must be prepared for its molecular identification, too.

Based on the result of the PFGE examination of the vancomycin-resistant strains, we suspect that the origin of vancomycin-resistant \textit{E. faecium} is monoclonal or at least one dominant strain could spread across Hungary. These strains were found predominantly in Western part of the country. The reason of this undoubtedly needs further investigation. The common sources might be the feed, environment, animal
breeders or the applied technology. On the other hand, the origin of vancomycin-resistant *E. durans* strains looked polyclonal. To further investigate this, we plan to run Long-range PCR examinations of the transposon.

Summarising the results, we have demonstrated that banning the avoparcin resulted in decreasing of the VRE strains in animal sources. Before VRE became a serious problem in Hungary the main reservoirs of these strains were discontinued and this might be the interpretation why are VRE fortunately so rare among the human isolates in Hungary.

The Antibiotic Monitoring System of animal isolates must be continued in Hungary and concentration of forces between human and veterinarian scientists is needed to liquidate VRE strains in Hungary, as well as to inhibit their spread into the human population, maintaining the advantageous situation we are currently facing in the country.

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