Epidemiology of *vanA* gene carrier enterococci: Molecular characterisation, antibiotic sensitivity and phylogenetic relationship of Hungarian isolates

Ágoston Ghidán
Semmelweis University, Institute of Medical Microbiology

Doctoral (Ph.D.) thesis

Semmelweis University Budapest, School of Ph.D. Studies
Pathology Interdisciplinar Doctoral School

Programme 8/3: Studies on the effects of microorganisms and their products at molecular, cellular and organism levels

Programme leader: Prof. Ferenc Rozgonyi, MD, PhD, DSc

Project leaders:
Prof. Sebastian G.B. Amyes, MSc, PhD, DSc, Dr*hc*, FRCPath, FIBiol
Prof. Ferenc Rozgonyi, MD, PhD, DSc

Official opponents:

Prof. Antal Szabó, MD, PhD, DSc
Miklós Füzi, MD, PhD

2007

TABLE OF CONTENTS

1. ABBREVIATIONS USED IN THE THESIS

ATCC	American Type Culture Collection
Atm	atmosphere
BHI	Brain-Heart Infusion
Bla^+	ß-lactamase-producing
bp, kb	base pairs, kilobase pairs
BSAC	British Society for Antimicrobial Chemotherapy
CFU	colony forming unit
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Ery	erythromycin
et. al.	and colleagues
GRE	glycopeptide-resistant enterococci
GI	gastrointestinal
HLGR	high-level gentamicin resistance
I	intermediate resistant
M, mM	mol/litre, millimol/litre
MHA	Mueller-Hinton agar
MIC	minimum inhibitory concentration
MIC_{50}	MIC, at which 50% of the tested isolates were inhibited
MIC_{90}	MIC, at which 90% of the tested isolates were inhibited
min, s	minute(s), second(s)
MRSA	methicillin-resistant *Staphylococcus aureus*
n	number
NCCLS	National Committee for Clinical Laboratory Standards
NCE	National Center for Epidemiology
NCTC	National Collection of Type Cultures
PBP	penicillin binding protein
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMSF	Phenylmethyl-sulphonylfluoride

R	resistant
RNA	ribonucleic acid
rpm	revolutions per minute
S	sensitive
sp.	specie
spp.	species
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UK	United Kingdom
UPGMA	unweighted pair group method with arithmetic averages
UTI	urinary tract ifection
UV	ultra-violet light
V	voltage
v/v	volume/volume
VRE	vancomycin-resistant enterococci
w/v	weight/volume

2. Introduction

2.1 The *Enterococcus* genus

The first vancomycin-resistant enterococci (VRE) that carried the *vanA* transposons were identified in 1986 in Europe (1, 2). Within 10 years VRE represented >25% of enterococci associated with bloodstream infections in hospitalized patients in the United States (3).

2.1.1. Characteristics of *Enterococcus* genus – Historical perspective

In 1899 the name "entérocoque" was first used in a paper from France (4). Later papers proposed that this organism was a hemolytic enterococcus (5). In 1906 the name *Streptococcus faecalis* (faecalis, referring to faeces) was used by Andrews and Horder, who isolated this organism from a patient with endocarditis (6).

In 1919 Orla-Jensen described *S. glycerinaceus* and *S. faecium* strains (7). These names were put aside and the organisms were considered to be the same as *S. faecalis* (5).

In 1937, it was acknowledged that the term enterococcus had been used to mean different things ranging from definition of any faecal streptococcus to a restricted definition of organisms that appeared to be identical to *S. faecalis* (5). An identification scheme was proposed, which separated streptococci into four divisions: pyogenic, viridans, lactic, and enterococcus. The enterococcus term was used for organisms that grew at 10 and 45°C, in 6.5% NaCl and at pH 9.6 and survived 60°C for 30 min; the ability to break down esculin was also highlighted (5). This scheme correlated well with the serological scheme, which exploited differences in cell wall polysaccharide antigens, introduced by Lancfield in the 1930s. In this test, the enterococci reacted with group D antisera, while pyogenic streptococci reacted with group A, B, C, E, F, or G and the viridans group were nongroupable. At that time, the recognized enterococcal species were *S. faecalis*, *S. zymogenes*, *S. liquefaciens* and *S. durans*. The basic biochemical reactions for identification were haemolysis and proteolysis. Some work had shown that the hae-

molysis is plasmid mediated and can be transferred to nonhaemolytic strains (8), even so it was considered to be a stable marker.

A number of studies in the 1940s and 1950s showed that the *S. faecium* described by Orla-Jensen in 1919, had biochemical characteristics that distinguished it from *S. faecalis.* Such differences were: inhibition by potassium tellurite, fermentation reactions, the lack of the ability to reduce tetrazolium to formazan (9, 10, 11).

The transfer of *S. faecalis* and *S. faecium* to the genus *Enterococcus* was proposed by Kalina in 1970 (12). Despite this, it was not achieved until 1984, when DNA-DNA and RNA-RNA hybridization studies showed a more distant relationship between these two species than other non-enterococal streptococci (13).

2.1.2. *Enterococcus* species

The genus was established in 1984 with the characterization of *Enterococcus faecalis* and *Enterococcus faecium*; however, a further 32 species have now been added to the genus on the basis of chemotaxonomic and phylogenetic studies (14). These additions were based on evidence provided by 16S rRNA sequencing studies. Members of the genus are listed in Table 1.

Table 1. List of *Enterococcus* species described

Current name: *Enterococcus*	Description of the species	Previous name
faecalis	Schleifer & Kilpper-Balz (1984)	*Streptococcus faecalis*
faecium	Schleifer & Kilpper-Balz (1984)	*Streptococcus faecium*
avium	Collins *et al.* (1984)	*Streptococcus avium*
casseliflavus	Collins *et al.* (1984)	*Streptococcus casseliflavus*
gallinarum	Collins *et al.* (1984)	*Streptococcus gallinarum*
durans	Collins *et al.* (1984)	*Streptococcus durans*
malodoratus	Collins *et al.* (1984)	*Streptococcus faecalis* subsp. *malodoratus*
hirae	Farrow & Collins (1985)	ND
mundtii	Collins *et al.* (1986)	ND
pseudoavium	Collins *et al.* (1989)	ND
raffinosus	Collins *et al.* (1989)	ND
solitarius	Collins *et al.* (1989)	ND
cecorum	Williams *et al.* (1989)	*Streptococcus cecorum*
columbae	Devriese *et al.* (1990)	ND
saccharolyticus	Rodrigues & Collins (1990)	*Streptococcus saccharolyti-cus*
sulfureus	Martinez-Murcia & Collins (1991)	ND
dispar	Collins *et al*. (1991)	ND
seriolicida	Kusuda *et al.* (1991)	*Lactococcus garvieae*
flavescens	Pompei *et al*. (1992)	ND
asini	de Vaux *et al*. (1998)	ND
haemoperoxidus	Svec *et al*. (2001)	ND
ratti	Teixeira *et al.* (2001)	ND
porcinus	Teixeira *et al.* (2001)	*Enterococcus villorum*.
moraviensis	Svec *et al.* (2001)	ND
gilvus	Tyrrell *et al.* (2002)	ND
pallens	Tyrrell *et al.* (2002)	ND
canis	De Graef *et al*. (2003)	ND
phoeniculicola	Law-Brown and Meyers (2003)	ND
italicus	Fortina *et al.* (2004)	ND
hermanniensis	Koort *et al*. (2004)	ND
saccharominimus	Vancanneyt *et al.* (2004)	ND

ND, New description

Source: http://www.bacterio.cict.fr/e/enterococcus.html (J.P. Euzéby : Dictionnaire de Bactériologie Vétérinaire)

Identifying *Enterococcus* species, comparative 16S rRNA sequence analysis has set up several 'species groups' within the genus (15, 16). There are currently eight species groups: the *Enterococcus avium* group (*E. avium*, *Enterococcus gilvus*, *Enterococcus hermanniensis*, *Enterococcus malodoratus*, *Enterococcus pseudoavium* and *Enterococcus raffinosus),* the *Enterococcus cecorum* group (*E. cecorum*, *Enterococcus columbae),* the group *Enterococcus dispar* (*E. dispar*, *Enterococcus asini, Enterococcus pallens),* the *E. faecalis* group (*E. faecalis*, *Enterococcus haemoperoxydus*, *Enterococcus moraviensis, Enterococcus ratti*), the *E. faecium* group (*E. faecium*, *Enterococcus canis*, *E. durans*, *Enterococcus hirae*, *Enterococcus mundtii, Enterococcus villorum*), the *Enterococcus gallinarum* group (*E. gallinarum, Enterococcus casseliflavus),* the *Enterococcus saccharolyticus* group with *E. saccharolyticus* and *Enterococcus sulfureus* group with *E. sulfureus.*

2.1.3. Identification of *Enterococcus* spp.

The classical *Enterococcus* species, *E. faecalis* and *E. faecium,* have numerous common characteristics on the basis they can be distinguished from other catalase-negative (although some strains do produce pseudocatalase), Gram-positive cocci. Most react with group D antisera and some react also with group Q antisera. These organisms do not have cytochrome enzymes. Hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR) is characteristic. They are able to grow in 6.5% NaCl and at pH 9.6, they grow at 10 and 45°C, and most survive at 60°C for 30 min (5, 13, 17). Many of the new enterococcal species fail to react with Lancfield group D antisera and fail to grow in the conditions that are characteristic of *E. faecalis* and *E. faecium* (18, 19, 20).

There are some other rapid routinely applied tests including aesculin hydrolysis, tests for urease, resistance to bile, β-glucosidase and β-glucoronidase activities, and an array of carbohydrate acidification test (14). Whilst none of these tests, or their combinations, are unique to enterococci, they remain valid tests to use when seeking only the classical *Enterococcus* species.

2.1.4. Natural habitat of enterococci

Enterococci are commensals of the gastrointestinal (GI) tract, being found in the intestine of nearly all animals, from insects to humans. In most healthy human adults in several studies from Japan, Germany and Scandinavia, enterococci were found in 97% (21, 22, 23, 24, 25). In faeces, they are typically found in high numbers: 10^5-10^7 CFU/g (26), being the predominant Gram-positive coccus in stools. They are also found in a number of environments, being readily recovered from a variety of outdoor sources: from vegetation and surface water, probably because of contamination by animal excrement or untreated sewage (27, 28).

Enterococci can also colonize the oral cavity and vaginal tract, although their recovery from these sites is relatively rare (< 20% of cases) (28).

2.2. Enterococcal infections

E. faecalis causes nearly 80% of human enterococcal infections, with the majority of the remainder caused by *E. faecium* (28). The interpretation of this disparity between the two species is the different abundance of *E. faecalis* and *E. faecium; E. faecium* viable counts in human faeces are, on average, 100-fold lower than those of *E. faecalis* (29). Another explanation for the preponderance of *E. faecalis* infections is that this species may have enhanced virulence over *E. faecium.* Many virulence factors have only been reported in *E. faecalis*. Studies from some locations, however, have reported that *E. faecium* is found more often than *E. faecalis* (30). Infections caused by enterococcal species other than *E. faecalis* and *E. faecium* are rare.

The main risk factor for enterococcal infection is the use of broad-spectrum antibiotics such as third-generation cephalosporins and other antibiotics with significant activity against anaerobes, including metronidazole, imipenem and clindamycin (31, 32, 33) which enables intestinal overgrowth of enterococci. Other risk factors for infection include prolonged hospitalization, a high severity of illness, intra-abdominal surgery and exposure to contaminated equipment or environmental surroundings (31, 32, 33).

The most common enterococcal infections are urinary tract infections, bacteraemia, endocarditis and intra-abdominal infections.

2.2.1. Urinary tract infections (UTIs)

The role of enterococci in urinary tract infections was first reported in 1906 (34, 35). In the last few years, the UTIs caused by enterococci has risen significantly, in some studies being reported as the second most common cause of nosocomial UTI (36). The increase in enterococcal UTIs is probably the consequence of increasing use of urethral catheters and broad-spectrum antibiotic therapy, as well as the increasing use of cephalosporins (37). The bladder, kidney and prostate are common infection sites, particularly in those patients with urinary tract abnormalities or indwelling catheters (26).

2.2.2. Enterococcal bacteraemia

Enterococcal bacteraemia is much more common than enterococcal endocarditis (38, 39). The data of nosocomial surveillance in the USA between 1986 and 1997 found enterococci as the third most common cause of nosocomial bacteraemia (12.8%) of all isolates (40). It is thought that enterococci can translocate across intact intestinal epithelia and lead to many bacteraemias with no identifiable source (41). Most cases of enterococcal bacteraemia have an identifiable source, including intravenous lines, abscesses, UTIs and contaminated hospital equipment (32, 33).

The use of broad-spectrum antibiotics is a primary risk for enterococcal bacteraemia, which leads to intestinal overgrowth of them (31, 33).

2.2.3. Endocarditis

Enterococci cause an estimated 5 to 15% of bacterial endocarditis. As with other enterococcal infections, most isolates are *E. faecalis*, however, other species can also cause this disease (42, 43, 44). Among isolates sent to the Centres for Disease Control and Prevention, endocarditis was the diagnosis given for patients from whom, beside *E.*

faecalis and *E. faecium*, *E. avium, E. casseliflavus, E. durans, E. gallinarum, E. raffinosus* were isolated (19). Enterococci are the third most common cause of infective endocarditis behind streptococci and *Staphylococcus aureus* (45).

2.2.4. Intra-abdominal infections

It is rare that enterococci alone cause intra-abdominal infections or soft tissue infections, and there is often only a limited ability to infect in pure culture (26). However, in mixed infections with an avirulent anaerobic microorganism the infection is much more severe (46). This microbial synergy between enterococci and anaerobes has been well investigated, despite the mechanism by which it occurs is not understood (46, 47, 48).

2.2.5. Other enterococcal infections

Enterococci are isolated from burn wound infections and infections of indwelling foreign devices such as intravascular catheters. Nosocomial enterococcal infection has also been reported in transplant patients (kidney, liver) (49). They are also known to infect other sites, including the central nervous system, lungs, ears and eyes, but these infections occur less frequently (26).

2.3. Antibiotic resistance in enterococci

In the last few years the rapid emergence of multi-drug resistant enterococci certified that they are major nosocomial pathogens. *E. faecalis* causes the vast majority of human enterococcal infections, it is with *E. faecium* where multi-drug resistance is characteristic (28).

Enterococci are intrinsically resistant to many antibiotics, but they are able to acquire antibiotic resistance determinants through the exchange of plasmids and conjugative transposons (50, 51).

Most enterococci are intrinsically resistant to cephalosporins, the anti-staphylococcal penicillins, aminoglycosides and clindamycin (52, 53). Trimethoprim-sulpha-

methoxazole demonstrated poor efficacy *in vivo* when compared with *in vitro* results (54, 55). The *in vivo* resistance caused by the ability of enterococci to utilize exogenous folates (56).

Enterococci have readily acquired plasmid or transposon-mediated resistance to various other antibiotics such as chloramphenicol, tetracyclines, macrolides and high-level resistance to clindamycin and (53). Target site alterations in *gyrA* and *parC* have rendered many available fluoroquinolones unsuitable for treatment of enterococcal infections (31, 57, 58).

The intrinsic and acquired resistance determinants are summarized in Table 2. Only very few reliable therapeutic options has been left for multi-drug resistant enterococci. In the case of serious enterococcal infection, the synergistic antibiotic combination is a cell wall-active agent (ß-lactam or glycopeptide) with an aminoglycoside is one of the few options left (59, 60).

Table 2. Summary of antibiotic resistance in enterococci

Antibiotic	**Mechanism of resistance**	**Origin of resistance**	**Responsible genes**
ß-lactam antibiotics	Low affinity of penicillin-binding protein	Natural	*pbp5*, *pbp5fm*
Aminoglycosides	Impermeability Aminoglycoside-modifying enzymes,	**natural:** low resistance, **acquired:** high resistance	*aac(6')-aph(2")*
Macrolides	Modification target, inactivation of drugs, efflux pump	Acquired	*ermA, ermB, ermC, mefA, mefE*
Fluoroquinolones	Low affinity of gyrase enzyme, efflux pump	Acquired	Mutation in *gyrA*, *parC*
Tetracyclines	efflux pump,	Acquired	*tet, otr*
Nitrofurantoin	Lack of nitrofuran reductase enzyme	Acquired	Mutation in *recA*
Teicoplanin	Low affinity to peptidoglycane	Natural Acquired	*vanA*, *vanD*,
Vancomycin	Low affinity to peptidoglycane	Natural Acquired	*vanA*, *vanB*, *vanC*$_1$, *vanC*$_2$, *vanE*

2.3.1. Penicillin resistance in enterococci

Enterococci, compared to streptococci, are relatively resistant to the penicillins because of the low affinity of the penicillin-binding proteins (PBPs) (52). The MICs of *E. faecalis* typically range from 1-8 mg/L, while *E. faecium* MICs are higher as the PBPs of *E. faecium* have lower affinities for the antibiotics than the PBPs of *E. faecalis* (28, 53). High-level penicillin resistance in enterococci can arise through two distinct mechanisms: ß-lactamase production and PBP overproduction.

2.3.1.1. ß-lactamase-producing enterococci

The first ß-lactamase-producing (Bla^+) strain of *E. faecalis* was reported in 1983 (61). The enterococcal penicillinase has subsequently been shown to be identical to the staphylococcal type A penicillinase, and has activity against penicillin, ampicillin and the ureidopenicillins (62). In antibiotic sensitivity tests, the levels of resistance conferred by enterococcal β-lactamases are very dependent on the inoculum size, so much so that at routinely used inocula (10^5 CFU/ml), Bla^+ enterococci are often no more resistant to penicillin than other enterococci (62). Increasing the inoculum to 10^7 CFU/ml, penicillin MICs can elevate to 1000 mg/L or more (62).

The ß-lactamase of enterococci is not released into the extracellular medium (61). The enzyme remains cell-associated, most likely due to either a failure to be cleaved from its signal peptide sequence, or binding of the enzyme to other cellular components (62).

2.3.1.2. Overproduction of penicillin-binding proteins (PBPs)

Non-penicillinase-producing, penicillin-resistant enterococci have been reported for decades and are usually *E. faecium.* Until recently, MICs of penicillin typically ranged from 8 mg/L to 64 mg/L (62).

The mechanisms involved in this resistance are overproduction of a low-affinity penicillin-binding protein and a further decrease in the affinity of one of these enzymes for penicillin (63). Overproduction may be associated with a deletion in a region upstream of the *pbp5* gene that ordinarily negatively controls synthesis of PBP5 (63).

2.3.2. Aminoglycoside resistance in enterococci

Enterococci are ordinarily relatively resistant to aminoglycosides due to impermeability, with gentamicin MIC values typically ranging from 4 to 64 mg/L (52). The combination of a cell wall-active agent with an aminoglycoside, however, provides a synergistic bactericidal effect that is most likely attributable to increased uptake of the aminoglycoside

in the presence of the cell wall-active agent (60). In addition to resistance mediated by impermeability, *E. faecium* is intrinsically aminoglycoside-resistant due to the activity of a chromosomal acetyltransferase enzyme that modifies the antibiotic (64).

Whilst high-level streptomycin resistance is sometimes mediated by alteration in ribosomal protein S12 (65), high-level aminoglycoside resistance is usually due to plasmid-encoded aminoglycoside modifying enzymes. Plasmid-encoded high-level streptomycin resistance (MIC >2000mg/L) was first identified in *E. faecalis* in 1970 (60). Such resistance, conferred by the production of streptomycin adenyltransferase, is now common in both *E. faecalis* and *E. faecium* (52). Nine years after the emergence of high-level streptomycin resistance, high-level gentamicin resistance (HLGR) was reported in *E. faecalis* (66), associated with the production of a bifunctional 6'-aminoglycoside acetyltransferase 2"- aminoglycoside phosphotransferase enzyme that confers resistance to all clinically-available aminoglycosides, with the exception of streptomycin (67). However, many HLGR strains also produce streptomycin adenyltransferase (52).

Other aminoglycoside modifying enzymes include a 4',4"-aminoglycoside nucleotidyltransferase that confers resistance to tobramycin, kanamycin, neomycin and dibekacin (68), and the gentamicin-modifying enzymes, encoded by the aph(2")-1b, aph(2")-1c, and aph(2")-1d genes, which confer clinically-significant gentamicin resistance (69, 70).

High-level aminoglycoside resistance is now common in *E. faecalis* and in *E. faecium*, with a prevalence typically ranging from 20-50% (71, 72).

2.3.3. The glycopeptide antibiotics

There are two clinically significant glycopeptide antibiotics in use today, vancomycin and teicoplanin. Vancomycin had been in clinical use since the 1950s, with its usage increased rapidly throughout the 1980s and early 1990s as it became recognized as sometimes the only reliable antibiotic for the treatment of multiply-resistant staphylo-

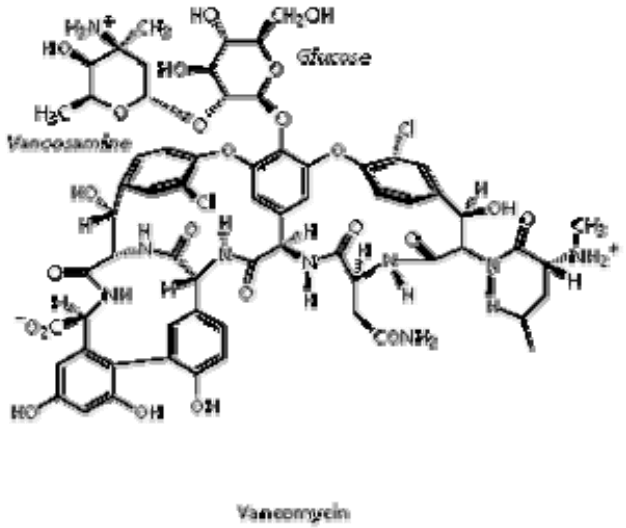
cocci (73, 74). However, vancomycin usage is associated with wellknown side effects, including ototoxicity, nephrotoxicity, neutropenia and severe allergic reactions (75). These disadvantages drove the investigation of other potential glycopeptide agents giving rise to teicoplanin, which is licensed for use in Europe. Both vancomycin and teicoplanin are produced naturally by the soil-living bacteria *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus* respectively (76, 77).

A third glycopeptide, which will be discussed later, is avoparcin. Avoparcin was used extensively throughout Europe for over 20 years as a growth-promoting agent in animal livestock production. Its use within the European Union was withdrawn in 1997 owing to concerns over a potential risk to human health. In Hungary, avoparcin was banned as growth promoter in 1998.

2.3.3.1. Activity spectrum of the glycopeptides

The glycopeptide antibiotics are large polar molecules and as such, cannot penetrate the outer membrane of Gram-negative organisms (78). Their activity is thus restricted to Gram-positive organisms, both anaerobes and aerobes.

The spectrum of activity of vancomycin and teicoplanin (Figure 1), although similar, is not identical (79). Teicoplanin is generally more active against streptococci and Gram-positive anaerobes than is vancomycin, whilst vancomycin has the greatest activity against coagulase-negative staphylococci. The main indications for glycopeptide usage are in the treatment of methicilli-resistant *Staphylococcus aureus* (MRSA) infections, *Clostridium difficile* infections, and serious Gram-positive infections in patients allergic to β-lactams.



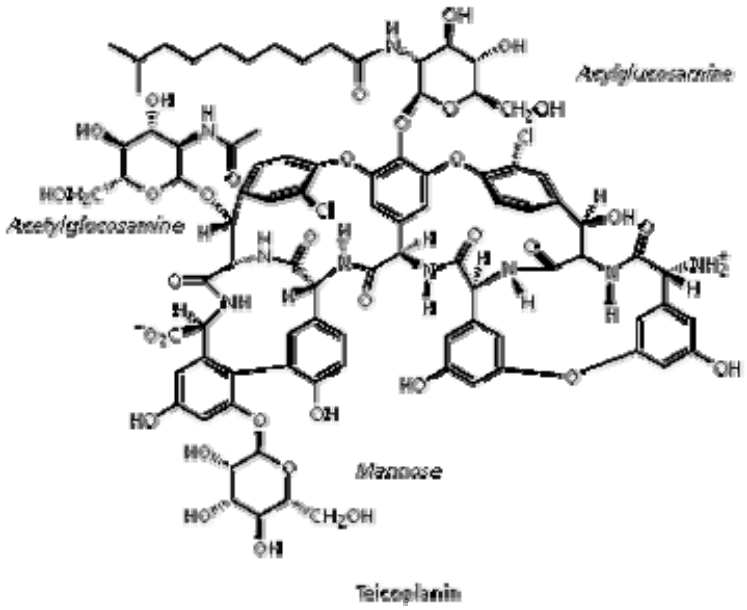


Figure 1. Structures of the glycopeptide antibiotics, vancomycin and teicoplanin (From Gilpin & Milner, 1997)

2.3.3.2. Mode of action of the glycopeptides

All glycopeptide antibiotics inhibit the latter stages of cell wall synthesis by forming complexes with peptidoglycan precursors. Within the cytoplasm of the cell, a D-alanyl-D-alanine (D-Ala-D-Ala) ligase produces the dipeptide D-Ala-D-Ala that is added to UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys (80, 81). Once the peptidoglycan precur-

sor is complete, it is translocated across the cytoplasmic membrane by a lipid carrier, and this stage is that the glycopeptides inhibit cell wall synthesis (82). Following translocation of the precursors to the outer surface of the cytoplasmic membrane, the glycopeptides bind to the carboxy-terminal D-alanine residues of the cell wall precursors. This binding blocks the incorporation of the peptidoglycan precursors into the nascent cell wall by transglycosylation, and leads to the accumulation of cytoplasmic precursors. Binding of the antibiotics to D-Ala-D-Ala-terminating peptide stems within nascent peptidoglycan is also believed to inhibit cell wall synthesis through inhibition of the transpeptidase and carboxypeptidase steps of cell wall synthesis.

2.3.3.3. Glycopeptide resistance in enterococci (GRE)

High-level resistance to the glycopeptides in clinical enterococcal isolates was first reported in 1988 (2). Further enterococcal isolates displaying a similar resistance phenotype were described later that same year (1). These two publications described what was to become known as VanA phenotype glycopeptide resistance, characterized by high-level inducible resistance to both vancomycin and teicoplanin. Whilst predominantly found in *E. faecium*, VanA-type resistance also occurs in *E. faecalis* and occasionally in other enterococcal species (83).

The year after the description of VanA-type glycopeptide resistance, a second phenotype was reported (84). This VanB phenotype is characterized by low to moderate levels of vancomycin resistance but susceptibility to teicoplanin, and is found predominantly in *E. faecalis* and *E. faecium* (83).

VanA and VanB phenotypes are the two most significant clinically forms of glycopeptide resistance in enterococci. However, since their description, other glycopeptide resistance phenotypes have been described. VanC phenotype glycopeptide resistance is an intrinsic property of *E. casseliflavus*, *E. gallinarum* and *E. flavescens*, and is characterized by low-level resistance to vancomycin and susceptibility to teicoplanin (85, 86). The recently described VanD, VanE and VanG phenotypes are acquired resistance traits seen in *E. faecium* and *E. faecalis* respectively (87, 88). These six resistance phenotypes are summarized in Table 3.

Table 3. Characteristics of the glycopeptide resistance phenotypes in enterococci

Characteristics	**Glycopeptide Resistance Phenotype**				
	VanA	VanB	VanC	VanD	VanE
Type of resistance	Acquired	Acquired	Intrinsic	Acquired	Acquired
Vancomycin MIC	64→>1000	8 → >1000	8→32	64→ 128	16
Teicoplanin MIC	16→> 1000	0.5→ 1	0.5→ 1	4	0.5
Expression	Inducible	Inducible	Constitutive	Constitutive	Inducible
Location of genes	Plasmid or chromosomal	Chromosomal or plasmid	Chromosomal	Presumably chromosomal	Presumably chromosomal
Transposon	Tn*1546*	Tn*1547*, Tn*3382*	-	-	-
Transferability	Yes	Yes	No	No	No
Terminal residues precursors	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Lac	D-Ala-D-Ser
Host species	*Enterococcus* spp.	*Enterococcus* spp.	*E. casseliflavus*	*E. faecium*	*E. faecalis*
		Streptococcus bovis	*E. gallinarum*		
			E. flavescens		
References	82	89	85, 86	88	87

2.3.3.4. Molecular basis of glycopeptide resistance

The resistance phenotypes (Table 3) share the same basic mechanism of resistance. The glycopeptides bind to the carboxy-terminal D-Ala residues of cell wall precursors, thus preventing their incorporation into the nascent peptidoglycan. Substituting the terminal D-Ala residue with either D-lactate (VanA, VanB and VanD phenotypes) or D-serine (VanC and VanE phenotypes) confers resistance (82).

The D-lactate (D-Lac) substitution causes the replacement of a peptide bond by an ester bond within the cell wall precursor. This replacement causes the loss of a single hydrogen bond that ordinarily forms between vancomycin and the cell wall precursor (90). The loss of this hydrogen bond results in a 1000-fold decrease in affinity for vancomycin binding, thus resulting in vancomycin resistance (90). The binding of vancomycin to the terminal D-Ala-D-Ala residues via hydrogen bonds is illustrated in Figure 2.

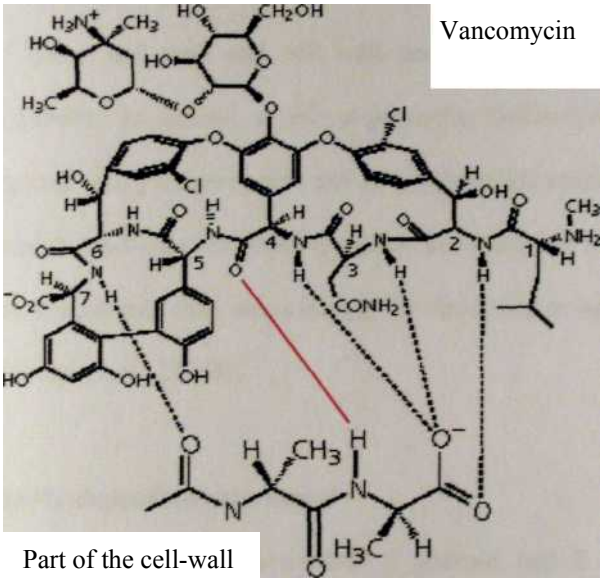


Figure 2. Binding of vancomycin to D-Ala-D-Ala, and the structure of the altered precursor, D-Ala-D-Lac

Hydrogen bonds formed between vancomycin and the terminal D-Ala-D-Ala residues

are illustrated, with the hydrogen bond that is lost by the substitution of D-Ala with lactate depicted in red (http://www.chemsoc.org/chembytes/ezine/1997/resist.htm).

In comparison with other antibiotic resistance traits that are conferred by a single gene product or mutation in a single gene, acquired glycopeptide resistance occurs by a complex mechanism involving a series of enzymatic reactions. VanA is conferred by seven different genes, involved in the regulation of resistance gene expression, the production of altered cell wall precursors (terminating in D-lactate) and the degradation of native cell wall and cell wall precursors (terminating in D-Ala) (82). In general, a cell co-producing D-Ala-D-Ala and D-Ala-D-Lac-terminating cell wall precursors will not be glycopeptide-resistant, as the antibiotic will bind to those D-Ala-D-Ala-terminating precursors as they are translocated across the cytoplasmic membrane, thus sequestering the lipid carrier necessary for their translocation (82).

2.3.3.5. Glycopeptide-dependent enterococci

In 1994, variants of glycopeptide-resistant *E. faecalis* and *E. faecium*, derived from both VanA and VanB phenotypes, were isolated from patients who had undergone vancomycin therapy (91, 92). These variants, known as glycopeptide-dependent enterococci, grow only in the presence of glycopeptide antibiotics or if supplied with the dipeptide D-Ala-D-Ala. This suggested that these variants are unable to produce the *ddl*-encoded ligase normally responsible for production of the D-Ala-D-Ala dipeptide. Recent studies have confirmed this, showing mutations in the *ddl* gene leading to either amino acid substitutions or deletions (93). With the impaired host D-Ala-D-Ala ligase, cell wall synthesis can only proceed in the presence of glycopeptides that induce the VanA or VanB ligase, resulting in synthesis of the D-Ala-D-Lac depsipeptide.

Vacomycin-independent revertants can arise from previously dependent strains through two distinct mechanisms. Compensatory mutations in the ddl gene can restore ligase activity, or alternatively, a mutation in the glycopeptide-resistance regulatory genes can result in constitutive expression of the VanA/VanB ligase (93).

Whilst glycopeptide-dependent enterococci have been described as 'superbugs' (94), it is generally accepted that they are more of a curiosity than a major clinical concern, carrying no more implication than normal GRE, with the exception that they are not readily detected by standard screening techniques.

2.3.3.6. Tn*1546* - the VanA transposon

The genetic element responsible for conferring high-level glycopeptide resistance in *E. faecium* BM4147, one of the first clinical GRE isolates, was fully characterized in 1993 (95). The element, designated Tn*1546*, is now known to confer VanA phenotype glycopeptide resistance, with all VanA enterococci harbouring Tn*1546*-related elements.

Tn*1546* (10,851-bp) is a Tn*3*-related transposon encoding nine polypeptides involved in the regulation of resistance, the resistance mechanism itself, and the transposition functions of Tn*1546*. The basic structure of Tn*1546* is well conserved and is depicted in Figure 3.

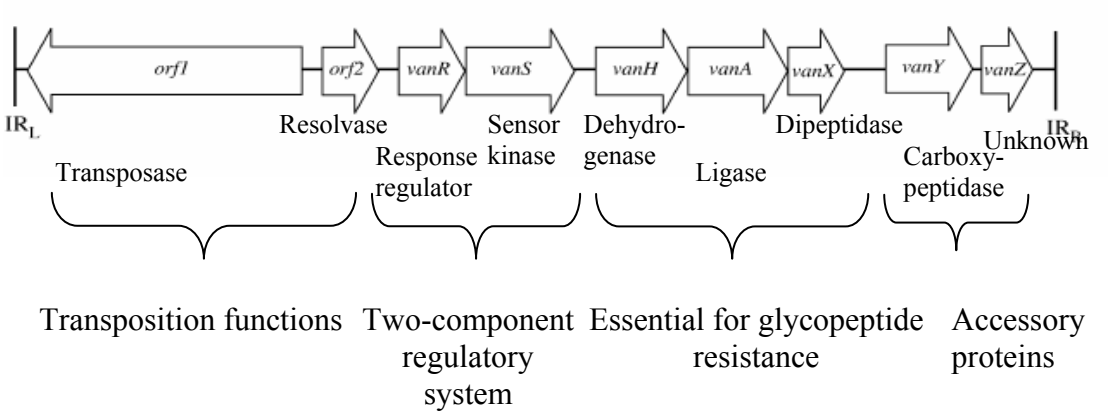


Figure 3. Genetic map of Tn*l546* transposon (95)

2.4. Therapeutic options for infections caused by glicopeptide-resistant enterococci

One implication of glycopeptide resistance in enterococci is that there may be no therapeutic agents commercially available and/or of established efficacy for patients with serious infection caused by multi-resistant *E. faecium.* Successful therapy currently depends on the antibiotic resistance profile of the particular infecting strain. Whilst it is sometimes possible to treat infections caused by GRE with 'traditional' antibiotics such as chloramphenicol (96), infections sometimes require treatment with experimental antibiotics or unproven antibiotic combinations. This has led to the rapid evaluation of numerous agents as potential replacements for vancomycin and teicoplanin.

2.4.1. Glycopeptide derivatives

Glycopeptide resistance arises through alteration of the drug target conferring cross-resistance to all naturally occurring glycopeptides. Thus it was widely thought that the glycopeptide class of antibiotics would be an unlikely source of potential replacements to overcome the problem of vancomycin resistance (97). Furthermore, unlike the β-lactam class of antibiotics, the glycopeptide molecule has limited potential for chemical modification (97). Recent attention, however, has turned to *N*-alkylated glycopeptides with these semisynthetic derivatives displaying high potency against VanA and VanB enterococci (97). These compounds have the unusual property of being bactericidal against enterococci, as well as exhibiting good activity against a range of Gram-positive organisms (97). The mechanism for their superior activity over their parent molecules has yet to be established.

2.4.2. Quinupristin-Dalfopristin

One of the non-glycopeptide alternatives to vancomycin for which there is the most clinical experience is quinupristin-dalfopristin, known as Synercid™, an antibiotic of the streptogramin class (97, 98). Synercid, licensed in late 1999, has received particular attention owing to its *in vitro* activity against a range of glycopeptide-resistant Gram-positive organisms, including VanA and VanB phenotype *E.faecium* (99). Its clinical

use may be limited, however, by its lack of activity against the predominant enterococcal species *E. faecalis* and its lack of bactericidal activity against *E. faecium* (97). Emergence of resistance to the quinupristin-dalfopristin combination during Synercid therapy of *E. faecium* infection has been reported (100), as has *E. faecallis* superinfection (101). Despite these problems, it is still thought likely that Synercid will prove a useful antibiotic for the treatment of infections caused by glycopeptide-resistant *E. faecium.*

2.4.3. Glycylcyclines

Whilst tetracycline resistance is common among enterococcal isolates, partly due to the widespread dissemination of Tn*916*-borne *tetM* gene, interest has focused on semisynthetic tetracycline derivatives which may have activity against those isolates resistant to the parent compounds (97). *N,N*-dimethylglycyiamido derivatives, referred to as glycylcyclines, have shown good activity against Gram-positive bacteria, including strains of multi-drug resistant *E.faecium* (65). These semisynthetic derivatives are poor substrates for the transporters mediating drug efflux and show greater affinity for ribosomes than the parent compounds, hence their activity against tetracycline and minocycline-resistant strains (102, 103). Like the parent compounds, however, the glycylcyclines are only bacteriostatic.

2.4.4. Fluoroquinolones

A large number of quinolones with enhanced activity against GRE, are undergoing development (97, 104). These newer compounds generally have much improved activity against enterococci than earlier class members, such as ciprofloxacin (105), and their bactericidal activity suggests they may be of use in the treatment of systemic enterococcal infections. Mutation studies with these newer fluoroquinolones have, however, demonstrated comparable mutation rates with those obtained with ciprofloxacin (105) and thus their therapeutic potential may be short-lived through the rapid acquisition of resistance.

2.4.5. Oxazolidinones

Oxazolidinones are novel synthetic, broad-spectrum, bacteriostatic agents that inhibit protein synthesis (97). They were initially investigated in the late 1980s but recent attention has focused on novel fluorinated oxazolidinones that demonstrate bactericidal activity against Gram-positive organisms, anaerobes and some Gram-negative species (106). No cross resistance to other antibiotic classes has yet been described and it is thought that these compounds show potential for the treatment of infections caused by various organisms, including GRE (97).

2.5. The threat of dissemination of vancomycin resistance genes

Arguably the main concern that was raised by the emergence of glycopeptide resistance in enterococci was the threat of its transfer to other organisms, particularly the 'first-rate' pathogens such as MRSA or penicillin-resistant pneumococci. The potential for the spread of the glycopeptide resistance genes has already been illustrated, with *vanA* being identified in various bacterial species, although not necessarily associated with Tn*1546*-like elements (107, 108).

Noble and colleagues have demonstrated the transfer of *vanA* into *S. aureus* under laboratory conditions (109), although the strains used were not MRSA. In the clinical setting MRSA appear to develop resistance to the glycopeptides by an alternative method: that of augmented cell wall production and PBP expression (110). Such MRSA strains exhibiting reduced susceptibility to vancomycin were first reported in Japan (111) and subsequently in Europe and the USA (112, 113), although they remain rare. Their clinical significance remains uncertain, as doubling the thickness of the bacterial cell wall is unlikely to be an efficient resistance mechanism, and the levels of glycopeptide resistance conferred are significantly lower than those mediated by VanA in enterococci.

Recently, for the first time in the history of this bacterial species, MRSA carrying the enterococcal *vanA* gene complex and expressing high level resistance to vancomycin

was identified in clinical specimens and was published in CDC, 2002 *MMWR* 51, 565–567.

2.6. Epidemiology of glycopeptide-resistant enterococci

Reports that followed the initial identification of acquired glycopeptide resistance in enterococci in the late 1980s were consistent with the glycopeptide resistance trait being another example of a nosocomial resistance trait, similar in many respects to methicillin resistance in *S. aureus.* Glycopeptide resistance was found predominantly, if not exclusively, in the nosocomial setting (114). However, European studies in the 1990s began to reveal a significant reservoir of GRE in various non-hospital sources and it is now recognized that the epidemiology of GRE is extremely complex and potentially influenced by several factors.

2.6.1. The European-American history of GRE

GRE have become a major infection control problem in the United States, far more so than in Europe (83). Despite their prevalence in US hospitals, no GRE have been isolated from community or non-human sources within the States (115), with the exception of one VanA GRE isolated from a sample of dry dog food (14) and one multi-resistant VanB GRE from animal feed (116). This is in stark contrast to the situation now being reported in many European countries. Whilst the scale of the problem of nosocomial GRE in Europe is not at the level being witnessed in the States, within Europe GRE are isolated from a variety of non-hospital sources including the community, pet and fern animals, raw meat and sewage (2, 117, 118).

A study in Belgium reported the recovery of GRE from 28% of community-based volunteers with no known hospital or glycopeptide exposure, the isolates being primarily polyclonal VanA (119). In the same report they describe the isolation of GRE from 64% of healthy volunteers who had been administered oral vancomycin or teicoplanin (119). No GRE were detected in the faecal flora prior to glycoside administration. As acquired glycoside resistance in enterococci does not arise through spontaneous mutation, these

findings suggest that, in Europe at least, GRE are frequent colonizers of the GI tract of healthy, non-hospitalized individuals, albeit in low numbers. These GRE may only reach detectable levels following glycopeptide therapy. It is therefore possible that GRE observed in the nosocomial setting within Europe actually have a community origin and are only seen following the intense selective pressures encountered in hospitals.

With the isolation of GRE from uncooked chickens and minced-meat products in Europe (120) it seems such meat and poultry products may act as a source for these community-based GRE. The ruggedness and heat tolerance of enterococci would be expected to enhance their ability for survival once having entered the food chain.

2.6.2. GRE in animal husbandry

2.6.2.1. *E. faecium* as an animal food supplement

A potential source of GRE in animal livestock that has received little attention is the use of *E. faecium* as an animal food supplement (114). *E. faecium* has a long history of use, being fed to livestock to stabilize and modulate the intestinal flora. The rationale behind its use is that *E. faecium* will confer colonization resistance, lactic acid and possibly bacteriocin production acting as a barrier to pathogenic bacteria. It is possible that GRE were among those *E. faecium* strains used as a food supplement. Prior to the initial description of enterococci with acquired glycopeptide resistance, the routine testing of clinical enterococcal isolates for this resistance trait was not widespread. It is possible, therefore, that *E. faecium* strains destined for use as a food supplement were not screened for glycopeptide resistance.

2.6.2.1. GRE and avoparcin

In the late 1960s, a report was commissioned to examine the use of antibiotics in animal husbandry and veterinary medicine (120). Among the recommendations of the report was that non-prescription use of antibiotics in animal feed should be restricted to those antibiotics which would not impair the efficacy of therapeutic antibiotics through the development of resistance (121). Despite this recommendation, the glycopeptide antibi-

otic avoparcin went into use as a growth-promoting agent in animal husbandry in 1975, even though resistance to avoparcin confers cross-resistance to vancomycin and teicoplanin.

Antibiotic growth promoters have been used in animal husbandry since the 1940s, particularly in the rearing of pigs and poultry (122). It is well established that animals reared in a germ-free environment grow faster than those reared in conventional surroundings (123). Coates *et al.* demonstrated that the growth rate of conventionally-reared animals can, however, be increased almost to that of the germ-free animals by administering low-level antibiotics continuously in their feed (123). The exact mechanism by which antibiotics improve growth and feed conversion efficiency is unknown, but it is thought that the subtherapeutic levels administered are able to control "low-level" diseases which would otherwise stunt the growth of the animals (122). All effects are limited to the intestinal bacteria, as the agents used are administered orally and are poorly absorbed.

Whilst it has been proposed that the low-level use of antibiotics as growth-promoters does not select resistant strains among the intestinal flora (124), there is increasing evidence to suggest otherwise. In Denmark, Aarestrup and colleagues reported high levels of vancomycin and avoparcin resistance among faecal isolates from animals (34). As vancomycin is not used therapeutically in veterinary medicine, they concluded that the use of avoparcin was responsible for the selection of GRE. Aarestrup *et al.* also proposed that, owing to the passage of avoparcin through the digestive system both largely unabsorbed and unmetabolized, the concentration of antibiotic in the GI tract is probably in excess of the avoparcin MIC for susceptible enterococci (34). Several studies have now reported a correlation between the use of avoparcin for growth promotion and isolation of VanA GRE from farm animals (118, 125). In the face of such findings, the use of avoparcin in the European Union was banned in 1997, owing to the potential risk it posed to human health through the selection of GRE.

The use of growth promoters has long been recognized as having economic advantages (122, 126) and it was therefore considered likely that alternative antimicrobial growth-

promoting agents would be used in place of avoparcin following its withdrawal (84). If resistance to these alternative antimicrobials, is linked to glycopeptide resistance genes, it could be anticipated that their use would continue to select GRE following the withdrawal of avoparcin (83). Early indications are, however, that avoparcin has led to a decreased incidence of GRE in animal livestock and the complete withdrawal of antimicrobial growth promoters in poultry production was not associated with economic loss (127).

Based on the above, there has certainly been the potential for GRE to be introduced and/or selected in animal livestock, with these GRE subsequently entering the food chain through the contamination of meat products. Following ingestion, these GRE strains may colonize the human gut or transfer their resistance determinants to the endogenous enterococcal flora. The relative importance of avoparcin use versus the clinical use of vancomycin and teicoplanin as factors promoting the selection and dissemination of GRE is still unclear. In the United States, avoparcin has never been licensed for use, a fact which may at least partly explain the lack of GRE in community and non-human sources in the US. The problem of GRE in the US is most likely due to the use and abuse of vancomycin in the clinics. The contrasting picture in Europe suggests avoparcin was certainly implicated to an extent in the widespread dissemination of GRE, but the clinical significance of its use remains uncertain.

3. Aims of this thesis

- To demonstrate the presence of VRE strains in Hungary

- To determine the presence of the VRE strains in healthy poultry in Hungary in a 4 year period between 2001-2004

- To determine the antibiotic resistance pattern of the strains from healthy poultry in that period.

- To establish the prevalence of *van* genotype of glycopeptide-resistant enterococci from animal and human sources in Hungary.

- To examine which Hungarian counties had the highest prevalence of VRE strains.

- To determine the heterogeneity of animal GRE by PFGE analysis, and to compare those GRE strains with human GRE isolates from hospital.

- To examine whether only one clone spread all over the country, or rather different types of strains emerged independently.

- Theoretical examination of the effect of the avoparcin prohibition in glycopeptide resistance in enterococci.

- To develop a DNA extraction protocol for PFGE examination

- To identify the *Enterococcus* spp. at species level by biochemical tests and by PCR method.

4. Materials and methods

4.1. Bacterial strains

4.1.1. Strains from slaughtered broiler chicken

The investigated animal strains in the study were isolated from slaughtered healthy animals within the confines of 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. In the case of broiler chickens we could meet the Office International des Epizooties' suggestion, as bacteria cultured from slaughterhouse samples should come from stocks of closely matched by age.

4.1.1.1. Isolation and identification of enterococci

Intestinal contents were diluted 1/10 (w/v) in a phosphate-buffered salt solution and a drop of this suspension was streaked on Enterococcosel (Becton Dickinson, Cockeysville, USA) agar plates. 0.5 ml of the suspension was inoculated into Enterococcosel Broth supplemented with 6 mg/L vancomycin. Both of them were incubated for 48 h at 37^{o}C in ambient atmosphere. Subcultures of the enterococcus-like colonies were made onto Slanetz-Bartley (Oxoid, Basingstoke, Hampshire, England) and Columbia blood agar (Merck, Darmstadt, Germany) 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* (8, 19). Identification of other colonies and isolates that showed growth on vancomycin-amended broth was performed to genus level by their phenotype as described by Devriese et al (128). Strains with special resistance patterns and vancomycin resistance were identified to species level (129). The vancomycin-resistant strains were identified at genus level by PCR (130) as well as the *van* gene carriers at species level also by PCR (131).

Bacterial strains were stored at -20°C in Brain-Heart Infusion (BHI) broth supplemented with 25% v/v glycerol. Stored strains were subcultured on to Blood-agar plates with a vancomycin disc to monitor stability of glycopeptide resistance. All enterococcal isolates gathered during the four-year period were screened for vancomycin resistance with 30 μg vancomycin discs (Oxoid Basingstoke, Hampshire, England). Any isolates tentatively identified as GRE were subjected to full glycopeptide susceptibility testing, as well as PCR and PFGE analysis.

4.1.2. Strains from human specimens

The first VRE strain in Hungary, an *Enterococcus faecalis* originated from the purulent discharge of an ulcer of the toe of a sixty-four year old diabetic male patient, admitted to a ward of the Department of Surgery, Nógrád County Hospital. Beside the traditional routine methods, identification was carried out by means of the Rapid ID 32 Strep kit (BioMérieux, Mercy-l'Etoile, France) according to the manufacturer's instruction. Antibiotic susceptibility testing was performed with an ATB STREP test (132).

The second VRE strain in Hungary originated from a 19-year-old man who had been admitted to the Petz Aladár Teaching Hospital in Győr, because of septic shock and large scale suffusions all over the body. The pathogen had proved to be *Neisseria meningitidis* serogroup C. In his stabilization period two superinfectious attacks arose. One of them was a bacteremia, caused by a vancomycin-sensitive *Enterococcus faecium*. The second was a wound infection in his deep colliquating necrotised tissue of the heel. Vancomycin-resistant *Enterococcus faecalis* (VREF) was isolated from this lesion with some Gram-negative opportunistic pathogens (133).

4.2. Materials

4.2.1. Media

Complex media used were BHI broth and agar, Enterococcosel (Becton Dickinson, Cockeysville, USA), Slanetz-Bartley (Oxoid, Basingstoke, Hampshire, England), Mueller-Hinton agar (MHA) and Columbia blood agar (Merck, Darmstadt, Germany). All were prepared according to the manufacturer's instructions, and were sterilized by autoclaving at 121°C/1atm for 15 minutes. All cultures were prepared in BHI medium unless stated otherwise.

4.2.2. Reagents

All chemicals and reagents were supplied by Sigma-Aldrich Company Ltd. (Budapest, Hungary) unless otherwise stated.

4.2.3. Antimicrobial agents

Antimicrobial agents used in this study are listed in Table 4. All were stored at 4°C with stock solutions freshly prepared as required. Antibiotics were dissolved in sterile ultra-pure deionized water alone.

Table 4. Antimicrobial agents used for agar dilution method and for determining the MIC

Antibiotic	**Commercial name/Manufacturer**
Vancomycin	VancocinCP/Lilly Hungária Ltd., Hungary
Teicoplanin	Targocid/Aventis Pharma Deutschland GmbH

4.2.4. Oligonucleotide primers

Primers were synthesized by Csertex Ltd., Budapest, Hungary. Primers were dissolved in sterile ultra pure deionized water in 50 nmol/ml concentration.

4.3 Methods

4.3.1. Antimicrobial susceptibility testing

Susceptibility of enterococci was tested with the disk diffusion test to ampicillin 10 μg , gentamicin 120 μg, streptomycin 300 μg, tetracycline 30 μg, erythromycin 15 μg, vancomycin 30 μg according to the NCCLS guidelines.

The minimum inhibitory concentrations (MICs) were determined by agar dilution test on MHA plates by BSAC guidelines (British Society for Antimicrobial Chemotherapy Working Party, 1991). Antibiotic stock solutions were prepared freshly at concentrations permitting the preparation of two-fold dilutions of antibiotic in MHA. Agar was allowed to cool to 50°C prior to addition of the antibiotic and, once set, the MIC plates were allowed to dry thoroughly, either at room temperature or for 15 minutes at 55°C.

Bacterial strains were subcultured from -20°C storage on BHI agar plates prior to inoculation into BHI broth. Following overnight incubation at 37°C, a 100-fold dilution of the overnight culture was made in sterile saline (0.85% sodium chloride). These diluted cultures were then used to inoculate the MIC plates with a Denley multi-point inoculator (Denley, Billinghurst, Surrey), resulting in approximately 10^4 CFU/spot. After allowing the inocula to dry, 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_{50} and MIC_{90} (concentration of antibiotic required to inhibit 50% and 90% of bacterial strains, respectively) were determined when appropriate.

4.3.2. Identification the GRE isolates by PCR

4.3.2.1. Extraction of DNA for the PCR

The DNA template for the PCR was prepared by heating bacterial cells (loop-full bacteria suspended in 0.1 ml sterile deionized water) to 99 °C for 15 minutes in a Perkin-Elmer (GeneAmp 9700, Norwalk, USA) 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.

4.3.2.2. PCR primers for identification

The control strains used for examinations are included in Table 5. The following primers were used for enterococcal sequences (Table 6): E1 and E2 binding to the 16S rRNA gene sequences. For species-specific identification the enterococcal superoxide dismutase (*sodA*) gene sequences were used. For *E. durans* DU1 and DU2, and for *E. faecium* FM1 and FM2 primers were applied.

Table 5. List of the standard bacterial strains used in the study as controls

Bacterial strains	**Resistance phenotype**
E. faecium ATCC 51559	VanA
E. faecalis ATCC 51299	VanB
E. gallinarum NCTC 12359	VanC-1
E. casseliflavus NCTC 12361	VanC-2

Table 6. Genus- and species-specific PCR primers and their product sizes and the control strains used

Primer	**Target genes**	**Sequence (5'-3')**	**Product size (bp)**	**Control strains**	**References**
E1	16S rRNA	TCAACCGGGGAGGGT	733	*E. faecalis* ATCC 29212	130
E2	16S rRNA	ATTACTAGCGATTCCGG			
DU1	*sodA*	CCTACTGATATTAAGACAGCC	295	*E. durans* ATCC 19432	*131*
DU2	*sodA*	TAATCCTAAGATAGGTGTTTG			
FM1	*sodA*	GAAAAAACAATAGAAGAATTAT	215	*E. faecium* ATCC 19434	131
FM2	*sodA*	TGCTTTTTTGAATTCTTCTTTA			
MU1	*sodA*	CAGACATGGATGCTATTCCATCT	98	*E. mundtii* ATCC 43186	131
MU2	*sodA*	GCCATGATTTTCCAGAAGAAT			

4.3.2.3. 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 described by Dutka-Malen and colleagues (134). To exclude the presence of the other *van* genes in the strains, they were examined also with the *vanB*, *vanC*$_1$ and *vanC*$_2$ primers (Table 7).

Table 7. The sequences of the *van* gene PCR primers

Primer	Sequence (5'-3')	Position
vanA1	GGGAAAACGACAATTGC	175-191
vanA2	GTACAATGCGGCCGTTA	907-891
vanB1	ATGGGAAGCCGATAGTC	173-189
vanB2	GATTTCGTTCCTCGACC	807-791
vanC$_1$*-1*	GGTATCAAGGAAACCTC	246-272
VanC$_1$*-2*	CTTCCGCCATCATAGCT	1061-1051
vanC$_2$*-1*	CTCCTACGATTCTCTTG	455-486
vanC$_2$*-2*	CGAGCAAGACCTTTAAG	885-869

The strains listed in Table 5 were used as positive control and *E. faecalis* 29212 (NCCLS quality control) was used as a negative control.

4.3.2.4. PCR thermal profiles

Template DNA (2.5µl) was mixed in REDTaq™ ReadyMix™ (Sigma-Aldrich). The master mix was prepared according to the manufacturer's instructions. The total reaction volume was 25µl. All amplifications were performed in a DNA Thermal Cycler (GeneAmp 9700). The thermal profiles are listed in Table 8. The PCR products were analysed by gel electrophoresis.

Table 8. The used thermal profile used for PCR amplifications

Primers	**Initial step °C-seconds**	**Number of cycles**	**Denaturation °C-seconds**	**Annealing °C-seconds**	**Extension °C-seconds**	**Final extension °C-seconds**
E_1-E_2	95-180	25	94-60	60-60	72-60	72-420
Du_1-Du_2	95-240	30	95-30	55-60	72-60	72-420
FM_1-FM_2	95-240	30	95-30	55-60	72-60	72-420
van genes	94-120	30	92-60	54-60	72-60	72-600

4.3.2.5. Analysis of DNA by agarose gel electrophoresis

PCR products were electrophoresed on 1.5% agarose gels in TAE buffer (40mM Tris-acetate pH 8.0; 2mM EDTA). Electrophoresis was performed on horizontal gel and the DNA samples were directly loaded into the gels. Samples were electrophoresed alongside a 100-bp DNA ladder (Sigma-Aldrich). Electrophoresis was performed at a constant voltage (100V) until the loading buffer fronts had moved to nearly the end of the gel. After electrophoresis, gels were stained in 0.5 mg/L ethidium bromide and visualized on a UV transilluminator. Photographs were taken with a digital camera system (Kodak DC-290 Zoom, Eastman-Kodak Co., Rochester New York) fitted with an orange filter.

4.3.3. 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. A loopful of bacteria was collected from blood agar after overnight cultivation and resuspended in 200µl Lysis buffer (6mM Tris-Cl, pH 7,6; 1 M NaCl, 100mM EDTA, pH 7,5 ; 0,5% Brij-58; 0,2% Desoxycholat; 0,5% N-lauroyl-sarcosine)

and 16,7µl (1 mg/ml) lysostaphin (Sigma-Aldrich) was added. 2% Low-Melting agarose (Bio-Rad, Hungary) was prepared with Lysis buffer, and after the bacterial suspension was mixed with agarose, the plugs were prepared immediately. When the agarose became solid, the plugs were immersed in 3ml of Lysis buffer. After incubation for 1 hour at 37°C, the buffer was changed to ESTN buffer (100mM EDTA, 10mM Tris, 1% N-lauroyl-sarcosine, pH 8) 10µl (20 mg/ml) buffer and Proteinase K (Sigma-Aldrich) was added and it was further incubated for 3 hours at 50°C. The buffer was changed to 2 ml TE buffer (10mM Tris-Cl, 1mM EDTA, pH 7,5) with 7 µl of phenylmethyl-sulphonylfluoride (PMSF) stock solution (35 mg of PMSF/ml of isopropanol) for blocking the Proteinase K and incubated for 30 minutes at 50°C. The plugs were then washed three times with TE buffer and the restriction with 10U of *Sma*I (Sigma-Aldrich) was performed at 25°C for 2 hours. Running parameters of the samples was defined by Murchan et al. (135): block switch 5-15s, 6V/cm, 10h and block 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.

4.3.3.1. Visual comparison of PFGE patterns

PFGE patterns were initially compared by eye, and interpreted by the criteria described by Tenover and colleagues (136). Using these criteria, isolates are deemed to be indistinguishable if they have identical PFGE patterns. Isolates differing by up to three bands are considered to be closely related, with four to six bands difference suggesting isolates are "possibly related". Isolates are considered unrelated if they differ by seven or more bands.

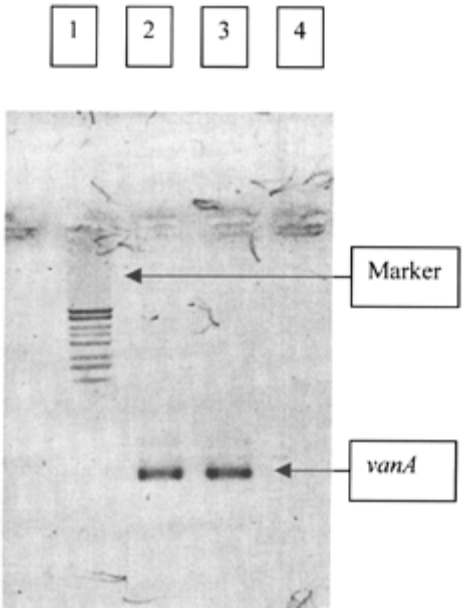
4.3.3.2. Computer-aided comparison of PFGE patterns

Photographs of gels were digitalized by a Kodak DC290-Zoom camera, banding patterns were compared and the dendrogram was set up by 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.

5. Results

5.1 Antibiotic sensitivity of the human *vanA* gene carrier strains in Hungary

The first VRE strain, confirmed by PCR (Figure 4), isolated in Hungary in 1998 had a minimum inhibitory concentration of vancomycin >256 mg/L and teicoplanin 96 mg/L.



Legend

Lane 1, molecular weight marker (10, 8, 6, 5, 4, 3, 2.5, 2, 1.5 and 0.5 kb); lane 2, *vanA*-positive control, ATCC 51559; lane 3, *E. faecalis* 6271/1998; lane 4, *vanA*-negative control, ATCC 29212.

Figure 4. Specific PCR amplification of *vanA* gene of the first vancomycin-resistant *Enterococcus faecalis* strain in Hungary.

The antibiogram of this strain is summarized in Table 9. The strain was sensitive to ampicillin, cotrimoxazole and rifampicin. Cefuroxime, erythromycin, lincomycin, vancomycin, teicoplanin, tetracycline and oxacillin were ineffective *in vitro* against the strain.

Table 9. Antibiotic sensitivity of the first VRE isolate in Hungary

Antibiotic	**Sensitivity**	**Antibiotic**	**Sensitivity**
Penicillin	I	Streptomycin HCl	I
Ampicillin	S	Tetracyclin	R
Oxacillin	R	Lincomycin	R
Ceftazidime	I	Nitrofurantoin	R
Cefuroxime Axetil	R	Rifampicin	S
Erythromycin	R	Cotrimoxazole	S
Gentamicin HCl	I	Vancomycin	R
Kanamycin HCl	I	Teicoplanin	R

Legends: S – sensitive, I – intermediate resistant, R – resistant

A strain isolated from the patient suffering from meningococcal meningitis in 2001 was the second *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 antibiogram is summarized in Table 10. The MICs of vancomycin and teicoplanin were 256 mg/L and 32 mg/L, respectively. PCR amplification with *vanA* primers revealed the presence of the *vanA* gene product from the strain.

Table 10. Antibiotic sensitivity pattern of *Enterococcus faecalis* strain isolated from the patient during the meningococcal meningitis

Antibiotic	**Sensitivity**	**Antibiotic**	**Sensitivity**
Penicillin	I	Tetracyclin	R
Ampicillin	S	Lincomycin	R
Erythromycin	R	Rifampicin	S
Gentamycin HCl	I	Co-trimoxazole	R
Kanamycin HCl	R	Vancomycin	R
Streptomycin HCl	I	Teicoplanin	R

Legends: S – sensitive, I – intermediate resistant, R – resistant

5.2. Antibiotic sensitivity by disc diffusion of the *Enterococcus* strains isolated from poultry in Hungary

During the examination period (2001-2004), the antibiotic susceptibilities of a total of 562 isolates were tested as shown in Table 11.

Table 11. Susceptibility rates of enterococci isolated from broiler chickens, 2001-2004

year	2001			2002			2003			2004		
n=	289			87			95			91		
%	S	I	**R**	S	I	**R**	S	I	**R**	S	I	**R**
amp	97.2	0	2.8	98.9	0	1.1	98.9	0	1.1	100	0	0
gen	100	0	0	100	0	0	100	0	0	100	0	0
str	96	1.4	2.8	90.8	1.1	8	93.7	0	6.3	98.9	0	1.1
tet	16.4	0.4	83.3	47.1	10.3	42.5	21.1	3.2	75.8	20.9	4.4	74.7
ery	39.7	11.9	48.4	51.7	23.0	25.3	25.3	29.5	45.3	35.2	24.2	40.7
van	64.7	11.8	**23.5**	73.6	14.9	**11.5**	85.3	7.4	**7.3**	91.2	6.6	**2.2**

Legends: S – sensitive, I – intermediate resistant, R – resistant, amp – ampicillin, gen – gentamycin, str – streptomycin, tet – tetracycline, ery – erythromycin, van – vancomycin

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 in 2003 and in 2004 in strains isolated from poultry. Among the isolates from chickens, tetracycline susceptibility was lower than 50%, in every year. The number of tetracycline-resistant and intermediate-resistant strains increased in 2003 and 2004. Among the broiler strains, 34 were intermediate susceptible, and 68 fully resistant to vancomycin in 2001. These numbers diminished to 6 and 2 in 2004, respectively (Table 12).

Table 12. The vancomycin susceptibility rates of enterococci isolated from broiler chickens determined by the disc diffusion method

Year	No of isolates tested	No (%) of strains		
		Sensitive	Intermediate	Resistant
2001	289	187(64.7)	34(11.8)	**68(23.5)**
2002	87	64(73.6)	13(14.9)	**10(11.5)**
2003	95	81(85.2)	7(7.4)	**7(7.4)**
2004	91	83(91.2)	6(6.6)	**2(2.2)**

5.3. Extraction of the DNA

5.3.1 Extraction of DNA for PCR

Several methods are published in international papers about the DNA extraction. Some protocols are too expensive, others are very time-consuming. In the beginning 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, but because lysostaphin was expensive, 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.

5.3.2. 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 uses only three buffers, therefore was used it 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.

5.4. Detection of the *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 *van* genes revealed that all VRE in this study were vancomycin-resistant because of the presence of the *vanA* gene, and no others – including *vanB* – could be detected. These strains were confirmed to be *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: *E. faecium*, *E. durans* and one *E. mundtii* strain. Figure 6, 7 and 8 are representative gels of the genus and species specific PCR for *E. faecium* and *E. durans*.

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

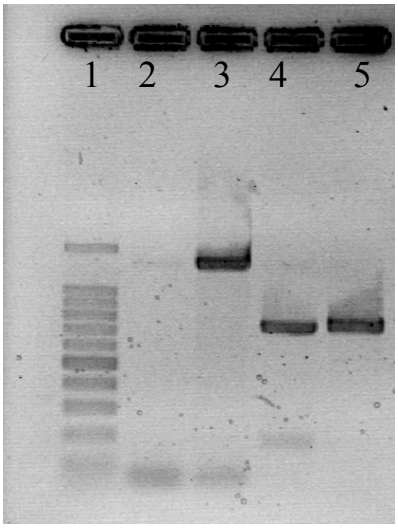


Figure 6. The genus specific identification of *Enterococcus* samples by PCR

Legends

1 – 100 bp. Marker, 2, 3 – not relevant to the present study, 4 – genus specific amplification, positive control of *E. faecalis* strain, 5 – genus specific amplification of an *Enterococcus* sp. strain

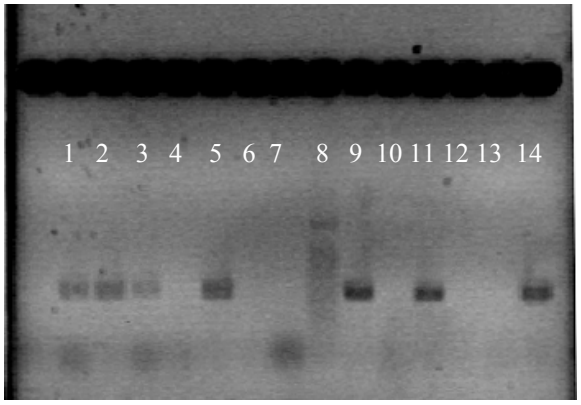


Figure 7. Species specific identification of *Enterococcus faecium* by PCR

Legends

1-3, 5, 9, 11, 14 – positive results: PCR product obtained; 4, 6, 7, 10, 12, 13 – no amplification: the strains are not *E. faecium,* 8 – 100 bp marker

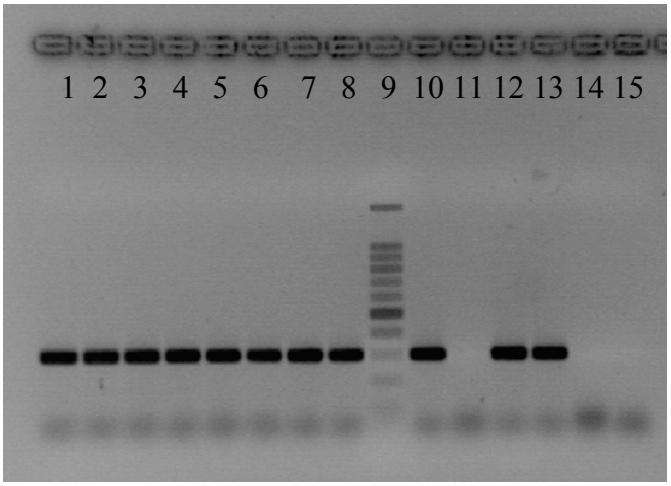


Figure 8. The species specific identification of the *E. durans* strains by PCR

Legends

1 – 8 abd 12 – 13: species specific (*E. durans*) amplification of the strains, 9 – 100 bp marker, 10 – positive control strain, 11 – negative control strain

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

The distribution of the MIC values of vancomycin and teicoplanin of the VRE strains isolated in 2001 and 2002 are summarised in Figure 8, 9, 10 and 11.

In 2001, 11 *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 *E. faecium* strain (11%) had an MIC of 64 mg/L (Figure 8).

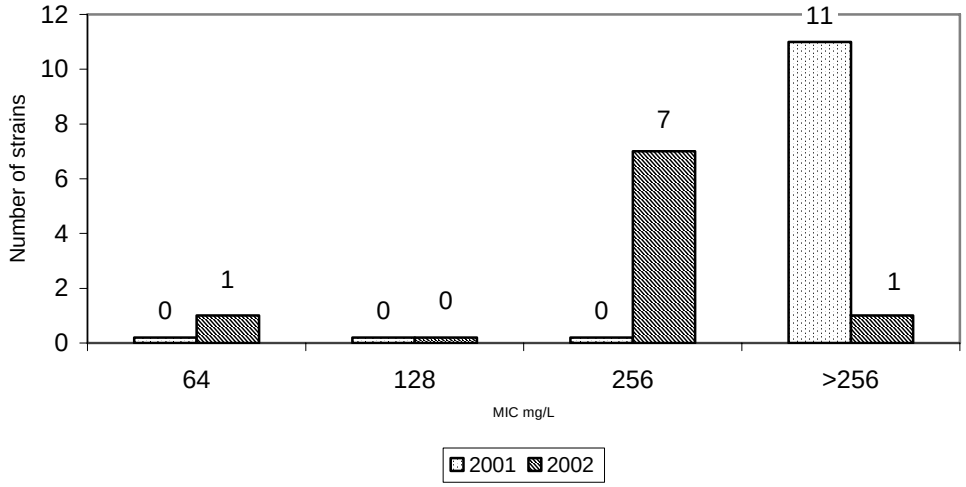


Figure 8. Distribution of the vancomycin MICs of the *vanA* positive *E. faecium* strains, isolated in 2001-2002

The distribution of the MICs of teicoplanin of the vancomycin-resistant *E. faecium* strains isolated in 2001 and 2002 was more diverse (Figure 9). In 2001, three strains (27%) had very high MICs of teicoplanin (>256 mg/L), one strain (9%) had 256 mg/L, 4 strains (37%) 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.

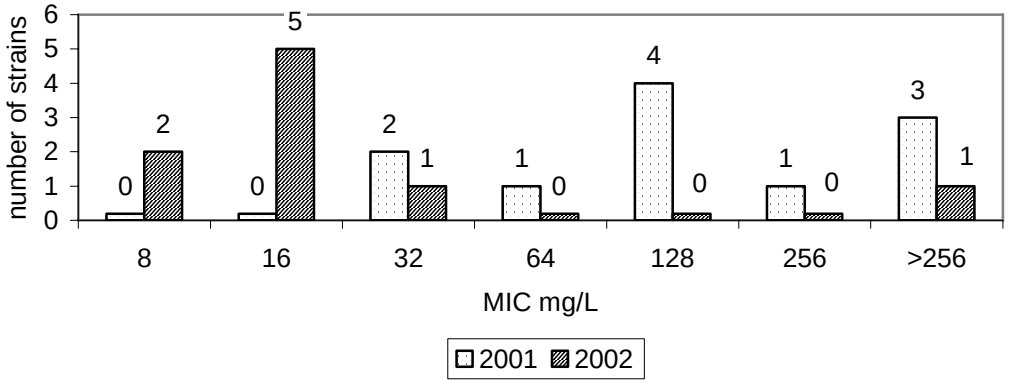


Figure 9. Distribution of the teicoplanin MICs of the *vanA* positive *E. faecium* strains, isolated in 2001-2002

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

The MICs of vancomycin of *E. durans* strains are summarized in Figure 10.

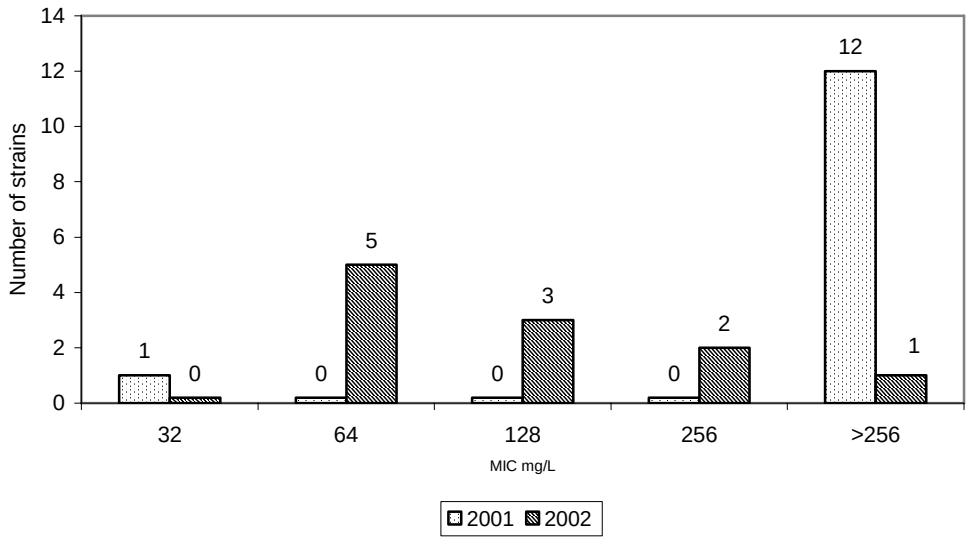


Figure 10. Distribution of the vancomycin MICs of the *vanA* positive *E. durans* strains isolated in 2001-2002

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 are shown on Figure 11. In 2001, 5 strains (38%) had higher MICs of teicoplanin than 256 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 and 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.

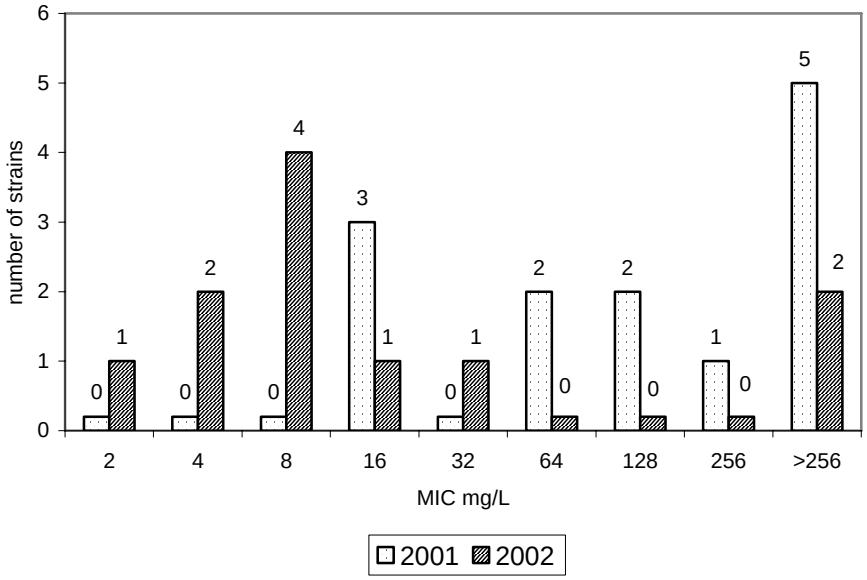


Figure 11. The teicoplanin MICs of the *vanA* positive *E. durans* strains isolated in 2001 and 2002

In 2003 and 2004, we could not find any *van* gene carrier strains.

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 oly 128 and 256 mg/L, respectively (Tables 13 and 14).

Table 13. MIC_{50} and MIC_{90} of the *vanA* positive *E. faecium* isolates (MIC given in mg/L)

Antibiotic	Year	MIC_{50}	MIC_{90}	MIC range
Vancomycin	2001	>256	>256	64 - >256
	2002	256	256	64 - >256
Teicoplanin	2001	128	>256	32 - >256
	2002	16	32	8 - >256

Table 14. MIC_{50} and MIC_{90} of the *vanA* positive *E. durans* isolates (MIC given in mg/L)

Antibiotic	Year	MIC_{50}	MIC_{90}	MIC range
Vancomycin	2001	>256	>256	32 - >256
	2002	128	256	64 - >256
Teicoplanin	2001	128	>256	32 - >256
	2002	8	>256	8 - >256

5.6. Geographical source of the VREs

The geographical source of the VRE strains at county level (Figure 12), isolated in Hungary between 2001 and 2002, is summarized in Figures 13 and 14.

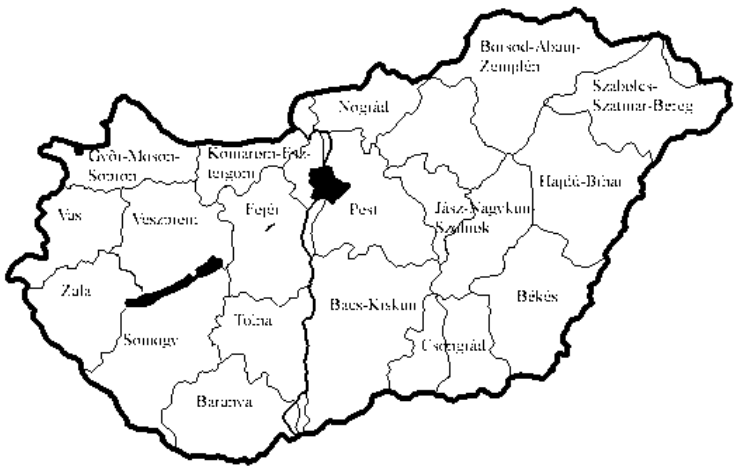
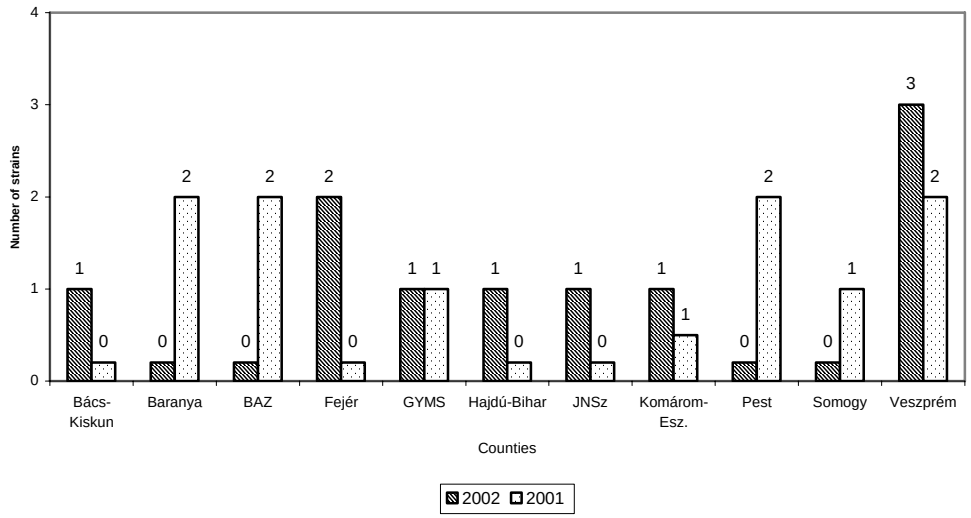


Figure 12. Counties of Hungary

In 2001, two *E. faecium* strains each 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 strains from Baranya, Borsod-

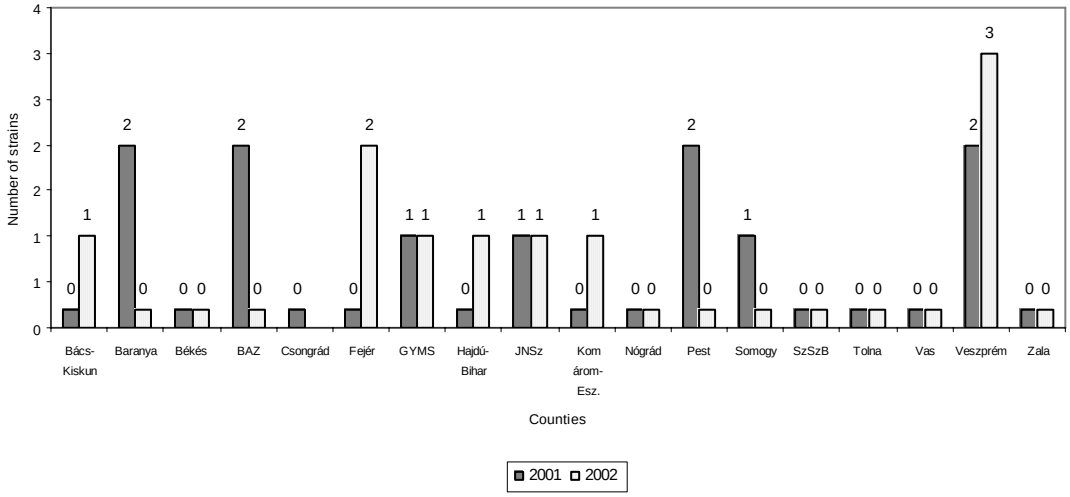
Abaúj-Zemplén, Pest, Jász-Nagykun-Szolnok and Somogy counties (Figure 13).



Abbreviations: BAZ – Borsod-Abaúj-Zemplén, GYMS – Győr-Moson-Sopron, JNSZ – Jász-Nagykun-Szolnok counties

Figure 13. Source of the Hungarian vancomycin-resistant *Enterococcus faecium* strains at county level in 2001 (n=10) and 2002 (n=11)

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 strains were isolated from Békés, Csongrád, Nógrád, Szabolcs-Szatmár-Bereg, Tolna and Zala counties. Vas county is an exception because in 2001 an *E. mundtii* VRE strain was isolated (Figure 14).



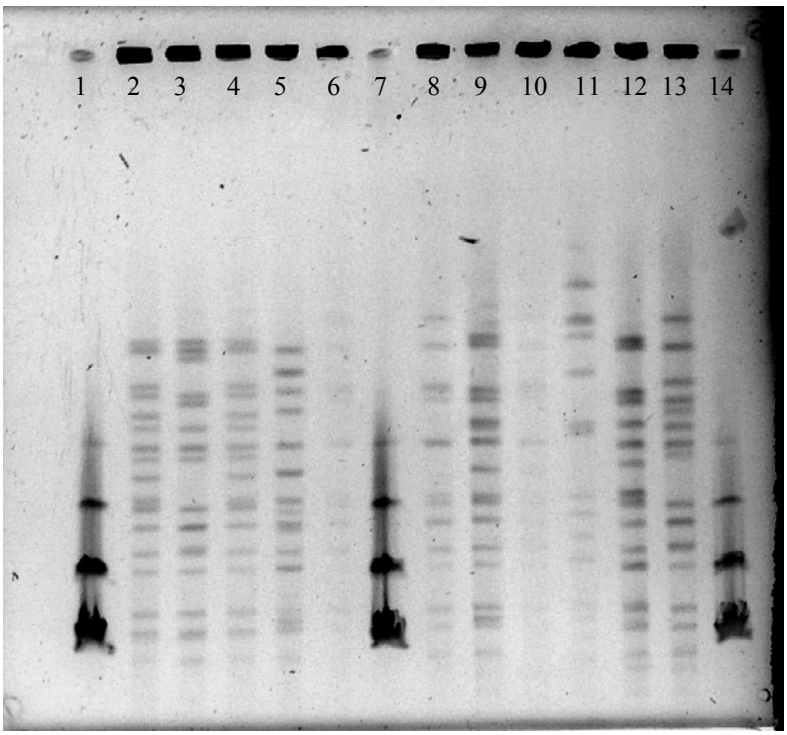
Abbreviations: BAZ – Borsod-Abaúj-Zemplén, GYMS – Győr-Moson-Sopron, JNSZ – Jász-Nagykun-Szolnok counties

Figure 14. Source of the Hungarian vancomycin-resistant *Enterococcus durans* strains at county level in 2001 (n=13) and 2002 (n=11)

5.7. 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 were offered a fully operating demo version of the Fingerprinting (Bio-Rad) 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. A representative PFGE gel is shown in Figure 15.

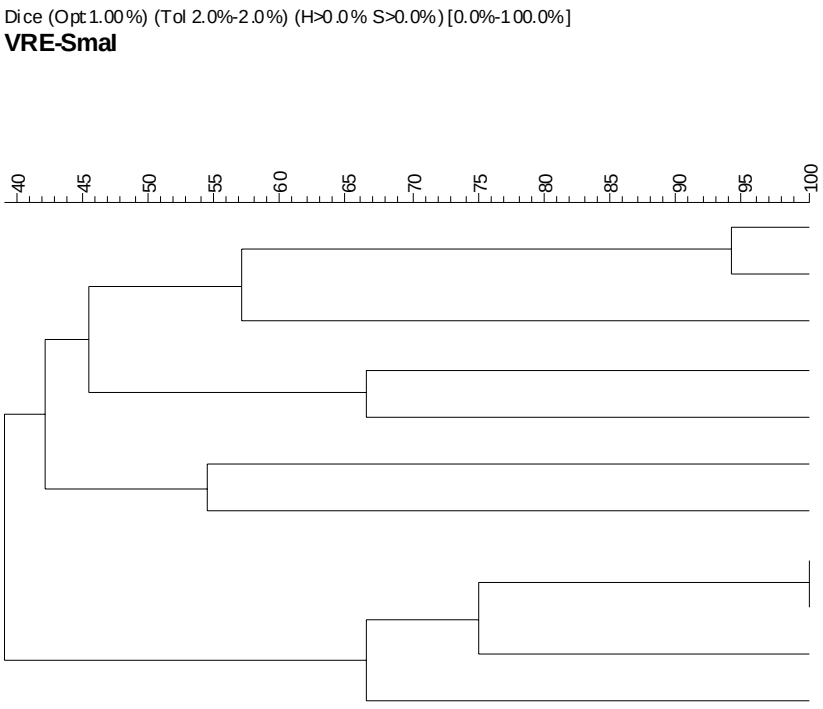


Legends

2 – 6, 8 – 13: VRE strains, 1, 7, 14: 50 kbp marker

Figure 15. A representative PFGE gel of the VRE strains

With this software the dendrograms were created according the species and isolation date. In 2001, eleven *E. faecium* strains were isolated with very high (>256 mg/L) MIC of vancomycin. Two identical strains were found in that year (Figure 16). One strain originated from Borsod-Abaúj-Zemplén and the other one from Pest counties. 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 demonstrate the polyclonal origin of vancomycin resistant *E. faecium* strains in that year.



Year	County	sp.	vanc MIC (mg/L)	teic MIC (mg/L)
2001	JNSz	E. faecium	>256	>256
2001	BAZ	E. faecium	>256	256
2001	Pest	E. faecium	>256	32
2001	Somogy	E. faecium	>256	128
2001	Baranya	E. faecium	>256	64
2001	Baranya	E. faecium	>256	128
2001	Veszprém	E. faecium	>256	128
2001	BAZ	E. faecium	>256	32
2001	Pest	E. faecium	>256	128
2001	Veszprém	E. faecium	>256	>256
2001	GYMS	E. faecium	>256	>256

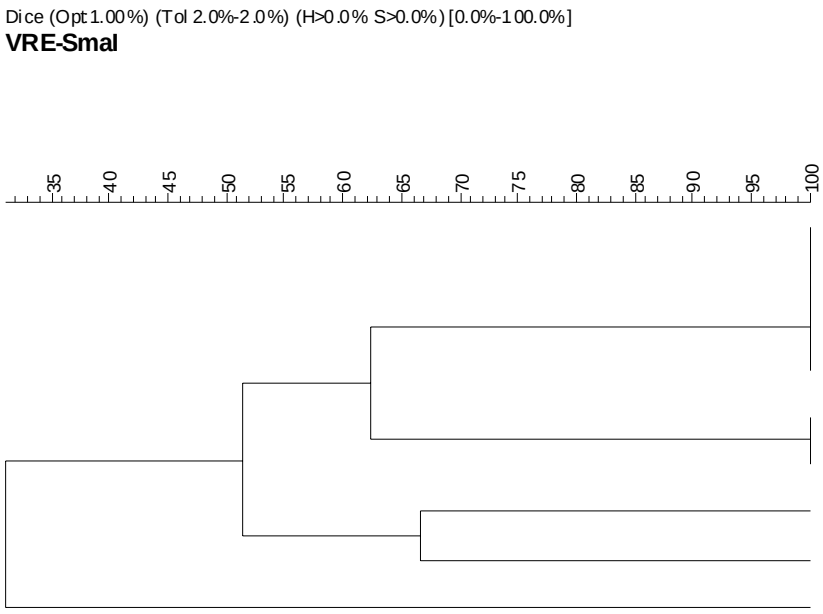
Figure 16. Dendrogram of vancomycin-resistant *E. faecium* strains isolated from chicken in 2001

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 (Figure 17). 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 each 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 (Figure 18), and the Eastern region of Hungary was also represented such 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.

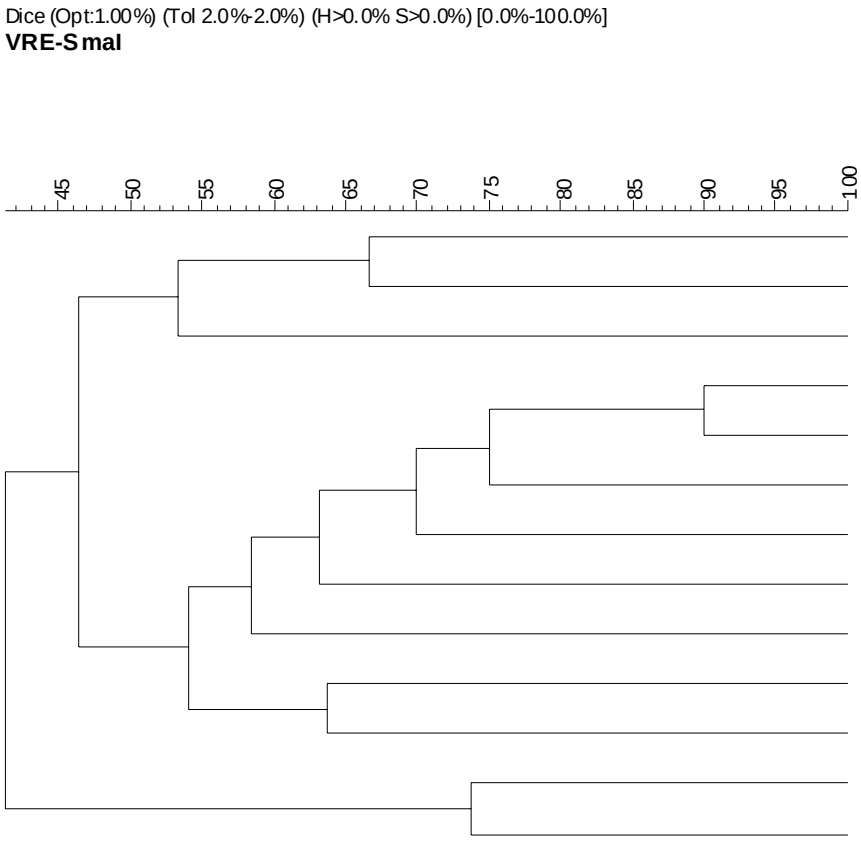
In 2002, 11 strains were isolated as vancomycin-resistant *E. durans* strains, but none of the strains were identical (Figure 19). Two strains were closely related (>90% similarity), both with an MIC of vancomycin of 64 mg/L. Their MICs to teicoplanin were 8 and 4 mg/L. The origin of these strains was Borsod-Abaúj-Zemplén and Hajdú-Bihar counties, both are located in the Eastern part of Hungary.

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.



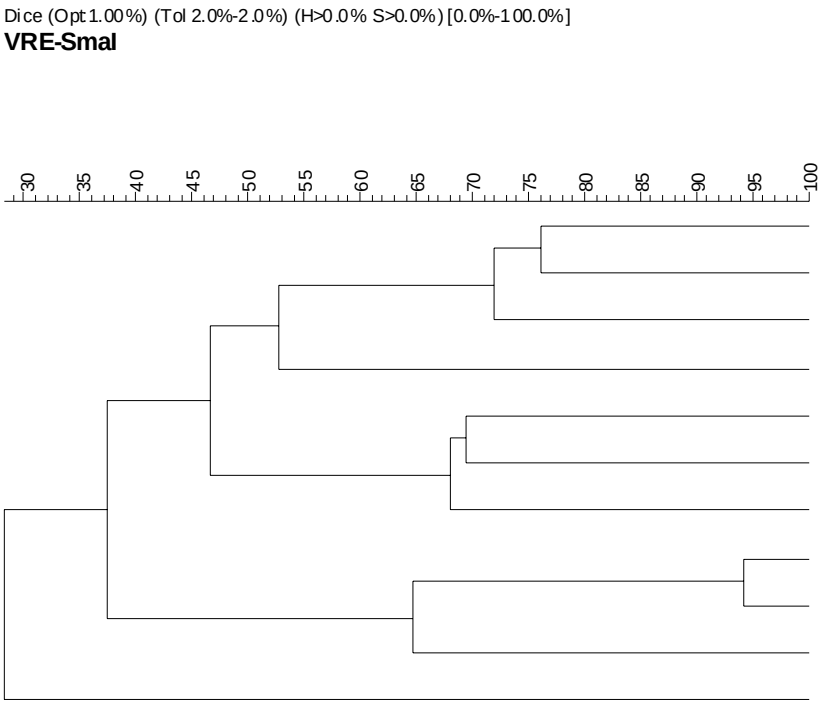
Year	County	sp.	vanc MIC (mg/L)	teic MIC (mg/L)
2002	Komárom.	E. faecium	256	16
2002	GyMS	E. faecium	256	16
2002	Bács-Kis.	E. faecium	256	16
2002	Fejér	E. faecium	256	8
2002	Veszprém	E. faecium	256	16
2002	Veszprém	E. faecium	256	16
2002	Fejér	E. faecium	256	16
2002	Veszprém	E. faecium	64	8
2002	Hajdú-Bi.	E. faecium	>256	>256

Figure 17. Dendrogram of vancomycin-resistant *E. faecium* strains isolated from chicken in 2002



Year	County	sp.	vanc MIC (mg/L)	teic MIC (mg/L)
2001	Tolna	E. durans	>256	>256
2001	JNSz	E. durans	>256	>256
2001	Bács-Kis.	E. durans	32	64
2001	SzSzB	E. durans	>256	>256
2001	SzSzB	E. durans	>256	128
2001	Baranya	E. durans	>256	128
2001	Békés	E. durans	>256	64
2001	Somogy	E. durans	>256	>256
2001	Komárom.	E. durans	>256	16
2001	Zala	E. durans	>256	256
2001	BAZ	E. durans	>256	16
2001	Nógrád	E. durans	>256	16
2001	Csongrád	E. durans	>256	>256

Figure 18. Dendrogram of vancomycin-resistant *E. durans* strains isolated from chicken in 2001



Year	County	sp.	vanc MIC (mg/L)	teic MIC (mg/L)
2002	Fejér	E. durans	256	32
2002	Tolna	E. durans	64	2
2002	Komárom.	E. durans	64	4
2002	Hajdú-Bi.	E. durans	64	8
2002	Hajdú-Bi.	E. durans	>256	>256
2002	BAZ	E. durans	128	8
2002	Baranya	E. durans	256	>256
2002	Hajdú-Bi.	E. durans	64	8
2002	BAZ	E. durans	64	4
2002	GyMS	E. durans	128	8
2002	Zala	E. durans	128	16

Figure 19. Dendrogram of vancomycin-resistant *E. durans* strains isolated from chicken in 2002

6. Discussion

6.1. Identification of enterococci

6.1.1. Identification of *Enterococcus* spp. by biochemical key

Strains isolated from non-human sources may be atypical and may not conform to the criteria used for standard phenotypic characterization. The ability of enterococci to grow under particular conditions is used in their selective isolation. These properties allow the detection and identification of enterococci with selective media (eg. Slanetz-Bartley agar, Enterococcosel agar) and by using bile-esculin-azide agar as a further test for confirmation (137, 138). Although this approach can distinguish *Enterococcus* spp. from other bacterial species, some isolates may be misidentified. These methods are unsuitable for the detection of certain enterococcal species because they fail to grow on these media (138). In addition, some other bacterial species (such as *Streptococcus bovis*) are able to grow on these media, so they may be misidentified as belonging to the *Enterococcus* spp. in the routine clinical laboratories. Several papers have been published on molecular methods (these have been based on the use of oligonucleotides) used for the detection of *Enterococcus* spp., however, the conventional methods for routine species identification are still based on physiological characteristics (139, 140, 141).

In the frame of the national annual surveillance in 2003 (National Center for Epidemiology [NCE], Methodological Letters) a total of 188 *Enterococcus* and 3 *Lactococcus* strains were examined by classical biochemical tests, such as group D antigen, bile-esculin reaction, growth in 6.5% NaCl broth and the pyrrolidonylarylamidase (PYR) test. The group D antigen test was highly discriminative between enterococcus and lactococcus strains because 77% of enterococci and none of the lactococci were positive. It is surprising that the Lancfield typing of enterococci was positive only in 77% of the cases. The bile-esculin test and the growth in the presence of 6.5% NaCl broth were unable to distinguish the *Enterococcus* and *Lactococcus*: in both cases both genera were positive in 100%. The PYR test gave 100% positive reaction for *Enterococcus* spp., but,

in a few cases (33%) was positive also for *Lactococcus* spp., so in these cases the strains would have been misidentified.

The biochemical tests needed for the identification and determination of *Enterococcus* spp. have been evaluated in studies which proposed tables or keys (19, 140, 142). However, the automated methods currently used are unable to reliable identify enterococci other than *E. faecalis* and *E. faecium* (26, 140, 143).

Based on the biochemical reactions available for the identification of *Enterococcus* spp. (144), our research group designed flowcharts that are summarized in Figures 21, 22, 23 and 24.

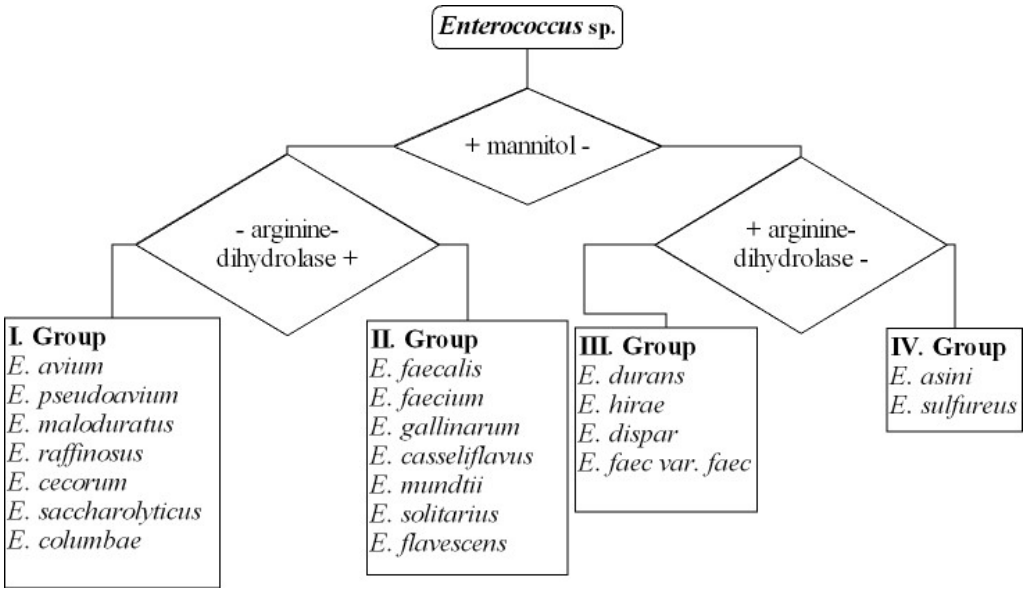


Figure 21. Identification of enterococci by biochemical reactions

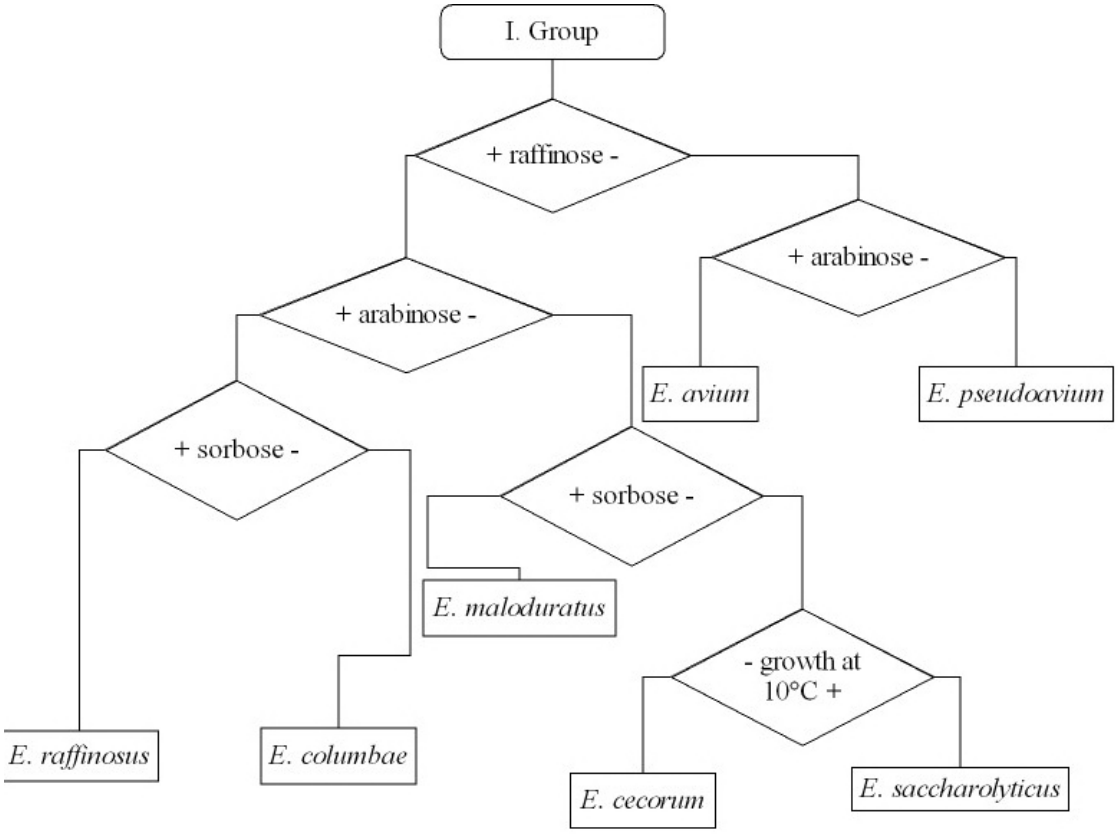


Figure 22. Identification of the members of group I. of enterococci by biochemical reactions

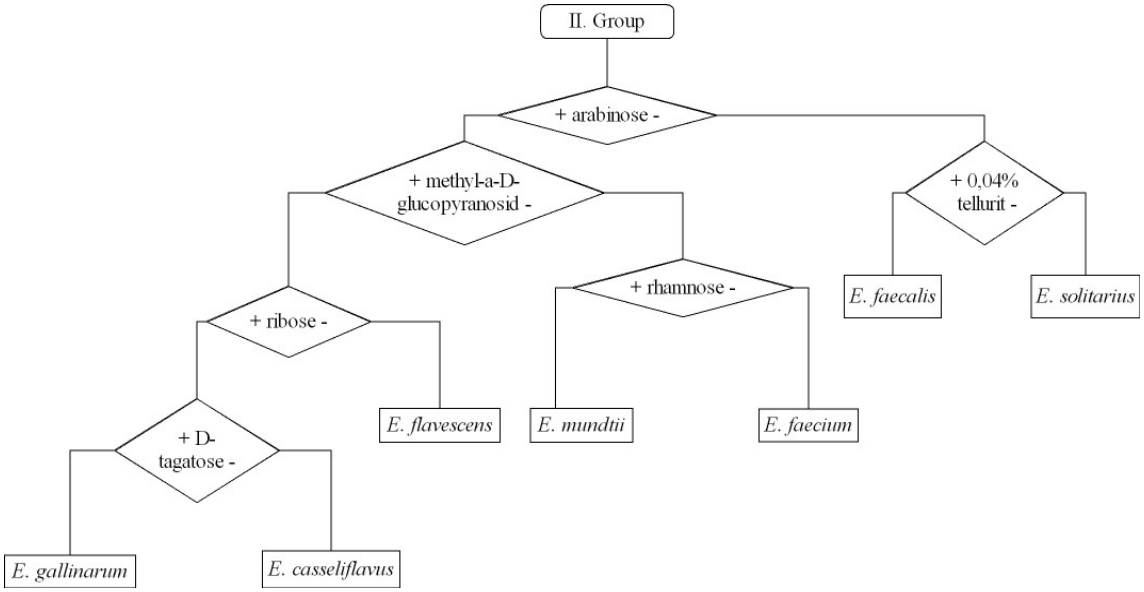


Figure 23. Identification of the members of group II. of enterococci by biochemical reactions

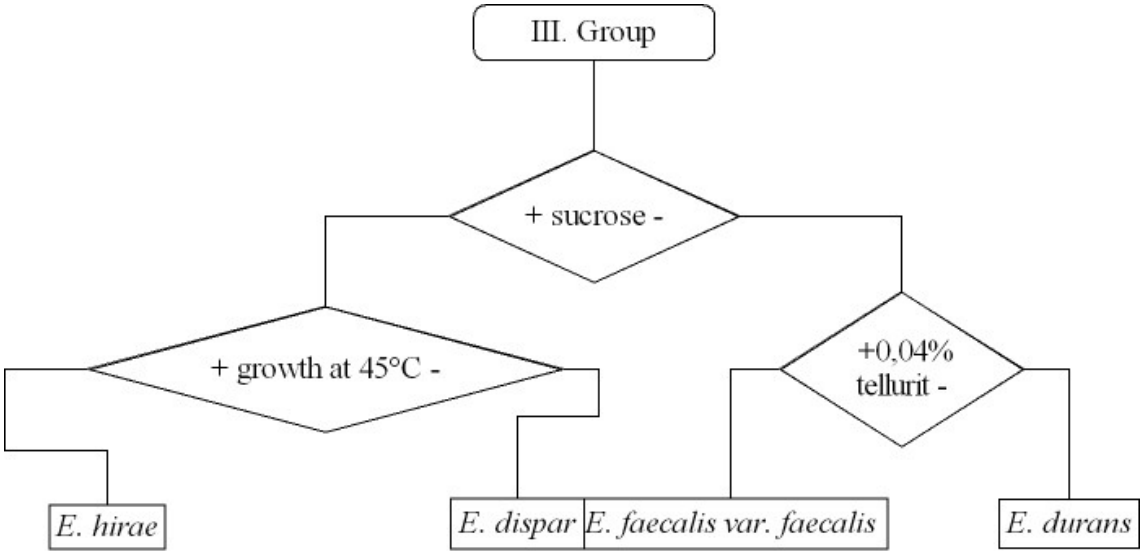


Figure 24. Identification of the members of group III. of enterococci by bio-chemical reactions

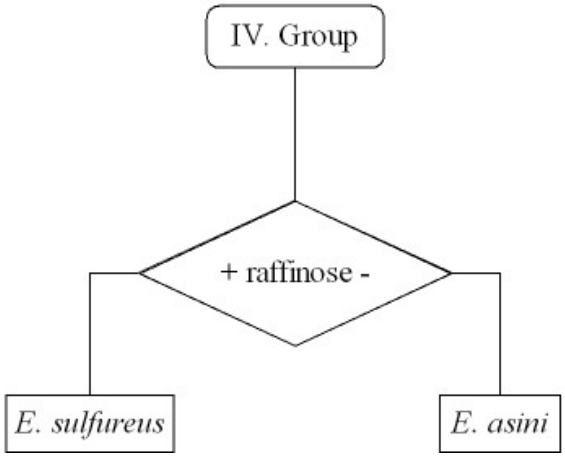


Figure 24. Identification of the members of group IV. of enterococci by biochemical reactions

These tests were selected to provide the greatest discrimination among *Enterococcus* spp., based on a high probability of a positive or negative result. Some of the tests could be performed with commercial kits (API 20 Strep or API 50CH), which are widely used (145, 146, 147). However, no commercial kit include the whole set of tests needed for the complete identification of *Enterococcus* spp. The advantage of these tests is the easy

use in diagnostic labs where a high number of isolates are examined, for rapid biochemical identification.

The comparative examination of the commercial API, BBL Crystal and Remel kits by the National Epidemiological Center laboratory (Methodological Letter) demonstrated that these tests are not sufficiently sensitive or discriminative for identification. Thirty-one strains were identified by API kit as *E. faecium*. The same strains in BBL Crystal Gram-positive ID kit test were identified as *E. faecium* in 28 cases and *E. casseliflavus*/*E. gallinarum* in 3 cases. When comparing the API kit with the Remel test, they demonstrated that out of the 43 strains, identified as *E. faecium* by API, only 22 strains were shown to be *E. faecium* with the Remel test, while 20 strains were *E. casseliflavus/E. mundtii/E. gallinarum* and one strain was identified as *E. gallinarum*. When a comparison was made between the BBL Crystal kit and the Remel kit, nearly the same discrepancies could be observed. Out of the 16 strains identified as *E. faecium* by BBL Crystal test, only 12 gave the same result by the Remel kit, in three cases they came up as *E. casseliflavus/E. mundtii* and in one case *Streptococcus* spp.

6.1.2. PCR assay for identification of *Enterococcus* spp.

Misidentification of *Enterococcus* spp., especially *E. faecium* isolates as *E. durans* (148, 149) or *E. gallinarum* (150) by automated systems or by conventional biochemical tests have been reported. The currently used automated methods are unable to identify reliably enterococci other than *E. faecalis* and *E. faecium* (151). Several studies were published on the problem of species identification (131, 143, 152). In these investigations various techniques were applied, including analysis of whole-cell protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (153), DNA hybridization, or contour-clamped homogeneous field electrophoresis (150), PCR amplification of the D-Ala:D-Ala ligase gene (133), and DNA hybridization in combination with pulsed-field gel electrophoresis (154). The sensitivity of these assays is unsatisfactory for direct detection from clinical specimens. A variety of conserved genes, including rRNA genes (155, 156, 157), the heat shock protein 60 (HSP60, CPN60) gene (158), the major cold shock protein gene (159) and the superoxide dismutase (*sod*) gene (160), have been exploited for the detection of the bacteria.

6.1.2.1 Genus- and species-specific PCR method for identification of *Enterococcus* spp.

The 16S rRNA gene has been used as target to develop genus-specific PCR primers for identification of enterococci. The *sodA* gene has been identified as a possible target for species differentiation of enterococci and degenerate primers were designed for amplifications. The primer sequences were not compared to sequences of other bacterial species sequences in the study we followed (131), because genus-specific primers were included in the PCR. Dicrepancies were reported between the PCR and the BBL Crystal kit (18%). Unlike the commercial kits, the PCR was able to identify as many as 23 different enterococcal species (131). The greatest agreement (90%) was observed between the PCR and VITEK test.

In a study in Italy (161) the phenotypic identification by multiplex PCR and 16S rDNA examinations were compared. Out of the 279 strains investigated, 26 isolates that were positively identified as *Enterococcus* spp. by biochemical properties could not be confirmed by 16S rDNA PCR. Six of these strains were either *Streptococcus* spp., *Lactobacillus* spp., or *Leuconostoc* spp. The strains identified as *E. faecium* by biochemical keys, were determined as *E. faecalis* by molecular examination.

Despite the different identification kits that are available, the classical biochemical tests are indispensable for enterococci and must be included in the identification protocol. Molecular examinations are unnecessary in everyday routine diagnosis and undesirable as they provide too much information; however, in serious diseases the diagnostic results at least must be confirmed by the PCR examination. If the strain is PYR positive, intermediately or fully resistant to vancomycin is able to grow on vancomycin screen agar and is a non-motile bacterium, then a molecular examination is necessary and probably obligatory (NCE, Methodological Letter).

6.2. VRE strains in Hungary

6.2.1 Human VRE cases in Hungary

Since the first report of VRE in Hungary in 1998 (132), very few cases have been reported and very few studies have been conducted to investigate the molecular epidemiology of such strains in this country (133).

In the national surveillance in 2003, 31 human isolates, intermediately or fully resistant to vancomycin were reported (NCE, Methodological Letter). However, the sensitivities were measured by disc diffusion and, only in a few cases, were the MIC of vancomycin determined. Much more accurate data can be gained if we compare the MICs of vancomycin. Based on this, only 23 strains were determined as non-susceptible: nine of them were fully resistant and 14 were intermediately-resistant to vancomycin. Five of the intermediately-resistant isolates were *vanC* gene carrier *E. casseliflavus* or *E. gallinarum* strains. (NCE, Methodological Letter).

Only one human VRE outbreak has been reported in Hungary and this occurred in the Haematology Centre of Budapest,(162). In this case all the strains were carriers of *vanB* gene. In the other hospitals, the prevalence of VRE – luckily from the point of view of the patients - is very low and very few strains have been available for analysis.

6.2.2 The presence of VRE in the Hungarian chicken population

Our study is the first in Hungary to perform molecular analysis of VRE strains isolated from healthy chickens in the abattoir. Our aim was to examine whether only one clone spread all over the country, or rather different types of strains emerged independently. Enterococcal samples (562) were collected from January 2001 to December 2004 from the intestine of healthy slaughtered chickens. In all, 46 strains (8.2%) were positive for *vanA*, and these were *E. durans* (52.2%), *E. faecium* (45.7%) and *E. mundtii* (2.1%). In comparison in an Italian study (163), where 25 isolates of animal origin were investi-

gated, the distribution of the species was: 17 (68%) *E. faecium* and 8 (32%) *E. durans*. Based on the international database, *E. faecium* and *E. durans* (sometimes *E. faecalis*) are the most common species isolated from chicken specimens (163, 164, 165, 166).

6.2.2.1. Vancomycin-resistant *E. mundtii* in the chicken population

Our research group found a vancomycin-resistant *E. mundtii* strain in a sample isolated from a chicken. This species has been reported only in very few cases as vancomycin-resistant, and mostly from environmental samples.

In a study conducted in South India, the prevalence of unusual (non-faecalis and non-faecium) enterococci and atypical (biochemical variant) species of enterococci in human isolates was determined as 19% (46 isolates) and 5% (12 isolates), respectively. The 46 unusual isolates were confirmed by phenotypic characterization belonging to the 7 following species: 15 *E. gallinarum* (6.2%), 10 *E. avium* (4.1%), 6 *E. raffinosus* (2.5%), 6 *E. hirae* (2.5%), 4 *E. mundtii* (1.7%), 3 *E. casseliflavus* - including the two atypical isolates - (1.2%) and 2 *E. durans* (0.8%). All strains were vancomycin and teicoplanin-sensitive (167).
These results serve as a warning to emphasize the importance of species identification and we must be aware that new species such *E. mundtii* can also harbour the *vanA* gene.

6.3. The *vanA* determinant in Hungarian chicken specimens

The *vanA* determinant was detected in various enterococcal species, including *E. faecium*, *E. durans* and *E. mundtii*, demonstrating that the *vanA* gene cluster located on mobile elements is able to disseminate between different species. According to the up-to-date literature, the *vanA* gene can be carried in *E. faecium, E. faecalis, E. avium, E. durans, E. gallinarum, E. casseliflavus, E. mundtii* and *E. hirae* (166). The most common carriers of this element are *E. faecalis, E. faecium* and *E. durans.* On the other hand, the *vanB* gene may be present only in *E. faecium* and *E. faecalis*.

Interestingly, the *vanB* determinant was not detected among the enterococcal strains during this study, although it was previously reported that the *vanA* mediated resistance

is the most widely distributed and predominant world-wide (168). The *vanA* gene cluster is located on the Tn*1546* transposon, which can be part of a conjugative plasmid (169, 170, 171). The *vanB* gene cluster may be carried on plasmid but it is usually located on the chromosome and the dissemination may be achieved by the conjugative transposon Tn*1547* (172, 173).

The *vanD* element has been only reported only in *E. faecium* so far (88). The presence of the $vanC_1$ gene is characteristic to *E. gallinarum*, whereas $vanC_2$ and $vanC_3$ can be present in *E. casseliflavus* and *E. flavescens* (86, 174). The *vanE* and *vanG* genes are very rare and have been detected only in *E. faecalis* isolates (87, 175).

6.4. The MICs of the VRE isolates

6.4.1. The vancomycin MICs of the *vanA* positive enterococci of human origin

The MIC of vancomycin of the first *vanA* positive human isolate in Hungary was 256 mg/L (132). Based on the published data, the MIC of vancomycin in *vanA* gene carrying strains vary between 16-1024 mg/L (166).

In the Hungarian study in 2003 (NCE, Methodological Letter), 23 human isolates were reported that were intermediately or fully resistant to vancomycin, and their MICs varied from 6 to >256 mg/L. Four strains had an MIC of 6 mg/L, six strains had 8 mg/L, four strains had 12 mg/L and seven strains had 32 mg/L. In two cases they measured MICs greater than 256 mg/L: these were identified as one *E. faecium* and one *Enterococcus spp*., both *vanB* positive. Five the other strains were *vanC* carriers *(E. gallinarum* and *E. casseliflavus)*.

In another study in 2001, 55 strains originated from four university hospitals in Korea were investigated (165), and 35 human clinical isolates of vancomycin-resistant *E. faecium* were found. All the strains were positive for *vanA*. The MIC_{50} and MIC_{90} of vancomycin were 512 and 1024 mg/L, respectively.

6.4.2. The vancomycin MICs of the *vanA* positive enterococci of chicken origin

All isolates carrying the *vanA* gene showed MICs of vancomycin of >256 mg/L in 2001, and 256 mg/L in 2002, respectively. In 2003 and 2004, we did not find any strains carrying *van* genes, only the MICs were slightly elevated (4-8 mg/L). These results coincided with the time period following the banning of the use of avoparcin in Hungary in 1998. The MIC_{50} and MIC_{90} of vancomycin of *E. faecium* strains were >256 in 2001 and 256 mg/L in 2002. The MIC_{50} and MIC_{90} of vancomycin for *E. durans* strains were >256 mg/L in both cases, while in 2002 the strains had an MIC of only 128 and 256 mg/L, respectively.

In a Korean study, the vancomycin-resistant *E. faecium* isolates had an MIC_{50} of 512 mg/L and an MIC_{90} of 1024 mg/L. Such high MIC values were found in human isolates, too (165).

Because these Hungarian strains originated from healthy chicken, it is evident that the virulence of the strains in this animal is low but that they constitute an important reservoir for the *vanA* determinant, which could eventually be transmitted to enterococcal strains colonizing the human gastrointestinal and genitourinary tract. A further important study, outside the scope of this thesis will be to survey poultry that have died due to enterococcal infections.

6.4.3. The teicoplanin MICs of the vanA positive enterococci of human origin

The first VRE strain found in Hungary had a teicoplanin MIC of 32 mg/L (132). The teicoplanin sensitivity of the human VRE strains is not well documented. Investigations have mostly focused on vancomycin resistance. In the specimens of hospitalised patients, the MIC of teicoplanin of the *vanA* positive VRE strains varied from sensitive to highly resistant category (166).

In a Swiss study (1998-1999) fecal samples of healthy employees from food industries were investigated. In a total of 50 specimens, 47 *vanA* positive *E. faecium* were isolated. The MIC of teicoplanin ranged from 32 to 1024 mg/L (176).

6.4.4. The teicoplanin MICs of the *vanA* positive enterococci of animal origin

The range of the MIC to teicoplanin in *E. faecium* in 2001 (n=11) was between 8->256 mg/L. The same range was found in 2002 (n=9), however, the distribution of MIC had moved towards the sensitive range. In the case of *E. durans* strains (n=13 and 11, respectively), a similar situation was observed, but the MIC range started with 2 mg/L. The MIC_{50} of teicoplanin among *E. faecium* and *E. durans* in 2001 was 128 mg/L, while in 2002 for *E. faecium* 16 mg/L and for *E. durans* 8 mg/L were observed.
In the above mentioned Korean study, where 20 *vanA* positive vancomycin-resistant *E. faecium* of poultry origin were investigated, the MIC_{50} was 16 mg/L (165).

6.5. Why are the MIC values of the *vanA* gene positive isolates so variable?

It has been known that the *vanA* gene cluster is carried as part of Tn*1546*-like elements, and this indicates that the horizontal transfer of the transposon plays an important role in the spread of VanA type resistance (177). The Tn*1546* element can be carried on plasmids or chromosomes. This element contains a polymorphic region, therefore many types of Tn*1546*-like elements can be distinguished by PCR mapping.

In the Korean study (165), the authors could find seven different types of the Tn*1546* transposon. The order of the genes of the Tn*1546* is: inverted repetitive sequences (IR), transposase (orf1), resolvase (orf2), *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, *vanZ* and IR. All poultry strains harboured this type of Tn*1546*–like element. The transposon found in human isolates contained one or more insertion elements (IS) such as IS*1542,* or IS*1216*. The IS*1542* was integrated between the *vanR* and *vanS* genes, while IS*1216*

was between the *vanX* and *vanY* genes. In this study the transfer rate of the *vanA* gene of the human and poultry VRE isolates was investigated. The rate of gene transfer in human VRE isolates were 10^{-4} to 10^{-5} per donor which was higher than those of the poultry VRE isolates (10^{-8} to 10^{-9} per donor).

The distribution of the insertion sequences associated with Tn*1546*-like elements among *E. faecium* from patients was investigated in Seoul (177). The isolates were divided into three main groups according to the distribution of ISs integrated into Tn*1546* elements. Type I was characterized by an IS*1542* insertion in the *orf2-vanR* intergenic region and an IS*1216V* insertion in the *vanX-vanY* intergenic region. Type II was characterized by two copies of IS*1216V* at the left end of Tn*1546*-like elements and in the *vanX-vanY* intergenic region, as well as IS*1542* in the *orf2-vanR* intergenic region. Type III was characterized by the presence of IS*19* in the *vanS-vanH* intergenic region and IS*1216V* in the *vanX-vanY* intergenic region (177).

In an Italian study (163) the *vanA* element was examined by combination of PCR and hybridization. A total of ten types of transposons, variously distributed among the three species (*E. faecalis, E. faecium* and *E. durans*) were identified, but none was common to animal and human strains. The strains had unrelated PFGE patterns.

The MICs of glycopeptide are so variable, because the Tn*1546*-like element is very variable, the function of the transposon is dependent of the presence of ISs. For example, Perichon *et al.* (178) reported that IS*19* was inserted in the D-Ala-D-ala ligase gene in the *VanD* positive *E. faecium* strain BM4416, resulting the inactivation of the *ddl* ligase.

The identification and characterization of *IS*s within the *vanA* gene cluster, analyzing and comparing the structures could be a useful tool in epidemiology.

6.6.The role of the avoparcin usage in vancomycin resistance

Glycopeptide derivates have been used outside the hospitals as growth promoters in animal husbandry. In particular, avoparcin, a glycopeptide antibiotic, was used as a feed additive for about 20 years in many European countries, including Hungary, as well as in Australia; however, it was never used in the U.S.A. and Canada. Farms using avoparcin became reservoirs for glycopeptide-resistant enterococci (179).

In European countries VRE could be detected from the feces of animals, and animal derived food (180). After discontinuing the usage of avoparcin as feed additive, the frequency of VRE in these reservoirs decreased significantly (181).

In contrast, in the United States, where avoparcin was not mixed into the food of animals, VRE have been isolated only from hospitalized patients and not from healthy individuals or animals. The other extreme example is Korea, where until 1998 avoparcin had been used to stimulate animal growth, VRE were isolated not only from hospitalized patients, but from animal feces and raw meat, too.

In Denmark about 24 tonnes of avoparcin was used for growth promotion in animals in 1994, while only 24 kg of vancomycin was used to treat humans (182). There is no information available in Hungary regarding how many kg of avoparcin for growth promotion was used and how many kg vancomycin were use for treating the serious infections of hospitalized patients.

Avoparcin was used as a growth promoter for broiler chickens in Hungary since 1989 but was banned in 1998. Due to this prohibition, they could not isolate any VRE in 2003 and 2004.

The farms that produced these strains can be reservoirs of VRE and the affected farms should change the technology of disinfection and breeding in order to prevent the emergence of high numbers of human VRE isolates in Hungary.

6.7. Genetic Relationship of the VRE strains

The use of molecular methods, such as PFGE, in the investigation resulted in the understanding of the epidemiology of VRE, allowing comparison of isolates from different sources. Recently, a multilocus sequence typing (MLST) scheme originally devised for *Streptococcus pneumoniae* (183) was proposed for *E. faecium* (184).

The PFGE patterns found in this study do not seem to be specific to a particular farm, county, or region of Hungary. By using PFGE, we demonstrated that the majority of the *E. faecium* and *E. durans* strains are, in general, not clonally related. Although PFGE is the standard typing method for enterococci, there are no standardized criteria for analyzing PFGE patterns. It has not been agreed how many band differences account for the description of a new clone, which is moreover true for long-term studies. PFGE demonstrated a high level of genetic diversity among *E. faecium* and *E. durans* population in farm environments in Hungary. Therefore, it would be useful to introduce long-range PCR for the detection of the diversity of the Tn*1546* transposon in epidemiological studies. While PFGE is able to determine the genetic relatedness of the strains, it is not suitable to detect the origin or the relationship of the vancomycin resistance genes. VRE isolates from different regions of Hungary are very polyclonal, suggesting the horizontal gene transfer of the vancomycin resistance genes rather than spreading of a single clone.

In our study, we detected vancomycin resistance genes in healthy poultry, despite avoparcin had not been used for many years. Although the use of avoparcin was banned in 1998, VRE strains could still be isolated for many years afterwards. However, it is clear that the number of the resistant strains and the MICs decreased continuously over this time period. The factors promoting the persistence of resistant bacteria or resistant genes in the absence of obvious selection pressure are not clear.

7. 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 identification, antibiotic sensitivity testing and genotyping of the isolates, using international guidelines and molecular methods. Because of the some uncertainties of theroutinely used methods, the identification of the strains was also confirmed by a molecular technique, the PCR amplification of the $ddl_{E.\ faecium}$ 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 *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 prohibition of avoparcin. On the other hand, 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 *vanA* gene in the Hungarian chicken population. Interestingly, there were 2 isolates with *vanA* gene found also in human isolates which is very rare. Recently *vanB* genes were found in the human VRE isolates.

Among chicken isolates were found vancomycin-resistant *E. faecium* and *E. durans* strains and only one *E. mundtii* strains. The number of publications of vancomycin-resistant *E. mundtii* is very low, this type of strain is very rare in environment, therefore the identification kits are not able to identify it at species level. According to our re-suls, 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 *E. faecium* is monoclonal or at least one dominant strain could spread across Hungary. These strains were found dominantly in Western part of the country. The demonstration of this undoubtedly needs further inves-tigation. 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 reservoires 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 contimued in Hungary and concentration of forces between human and veterinarian scientists is needed to liq-uidate thre 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 coun-try.

8. SUMMARY

The thesis is discussing the examination of the vancomycin-resistant *Enterococcus* (VRE) strains. The VRE isolates of animal origin derived from the Central Veterinary Institute, collected continuously at the abattoirs in the frame of the Antibiotic Monitoring System between 2001-2004, from the intestine of healthy poultry (broiler chickens). Avoparcin as a growth promoter was used for many years in animal feeding, creating a selective pressure on the normal intestinal flora of the animals. Among the obtained samples, most VRE strains were isolated from poultry, especially from broiler chickens. We have examined these strains in the study, with both conventional as molecular methods. Despite the ban on the use of avoparcin in Hungary since 1998, the animals are still carrying VRE strains, with the possibility of entering also to the human population. These strains can serve as a source of glycopeptide resistance which can be transmitted to even much more pathogenic bacteria. This phenomenon is not unknown and was published several times. We have identified the isolates at genus and species level with molecular methods, adapting the protocols described in the international literature to the local situation. The glycopeptide MICs of the positively identified strains were determined by the agar dilution method, using a multipoint inoculator. Most of the strains were resistant to both vancomycin and teicoplanin. Out of the examined resistance determinants, we found only the presence of the *vanA* gene by PCR, which is located on the Tn*1546* transposon.

It is worth to ponder that although these animals are not exposed to glycopeptides, why are the *Enterococcus* strains, found in their intestine resistant? All isolates carrying the *vanA* gene, from both animal and human origin, were compared at molecular level. As human VRE are fortunately still extremely rare in Hungary, we could involve only two human isolates in the study.

We detected the presence of the *vanA* gene in the animal isolates in 2001 and 2002, but in 2003 and 2004 we found only intermediately vancomycin-resistant strains, that

did not carry any of the common *van* genes (*vanA, vanB, vanC*). During the species determination, we could identify not only the most frequent species, but on one occasion we managed to identify an *Enterococcus mundtii*, which is a very rare *vanA* carrier, even according the literature.

The results of the phylogenetic examinations support the predominantly polyclonal origin of animal isolates, as well as the lack of their relatedness with human strains. It would be necessary to perform a thorough surveillance to screen for the source of resistance at those farms, where even five years after the ban of avoparcin VRE strains could be isolated, and if necessary, even the poultry-breed technology could be altered in cooperation with the farmers.

9. ÖSSZEFOGLALÓ

A dolgozat a vancomycin-rezisztens *Enterococcus* (VRE) törzsek vizsgálatát tárgyalja. Az állati eredetű VRE törzsek az Országos Állategészségügyi Intézetből származtak, amelyeket folyamatosan gyűjtöttek az Antibiotikum Monitoring Rendszer keretén belül, 2001-2004 közötti időszakban vágásra kerülő baromfiak (brojler csirkék) beléből származtak. Az avoparcint mint hozamfokozót éveken keresztül használták a takarmányozás során, ami szelekciós nyomást gyakorolt az állatok béltraktusában élő baktériumokra. A beérkezett minták közül a baromfiakból és ezen belül a brojler csirkékből lehetett a legtöbb VRE törzset izolálni. A továbbiakban ezeket a törzseket vizsgáltuk hagyományos és molekuláris módszerekkel. Az avoparcin etetése 1998 óta tilos Magyarországon, mégis az állatok VRE törzseket hordoznak, amelyek átkerülhetnek az emberekbe is. Ezek a törzsek glycopeptid rezisztencia forrásaként szerepelhetnek és akár sokkal pathogénebb baktériumokba is kerülhetnek. Ez a jelenség az irodalomban igazolt. A törzseket genus és species szinten meghatároztuk molekuláris módszerekkel. A nemzetközi irodalomban közölt módszereket adaptáltuk a helyi viszonyokhoz. Az identifikált törzsek glycopeptid MIC értékét agardilúciós módszerrel ún. multipoint inoculator segítségével határoztuk meg. A törzsek többsége rezisztens volt vancomycinre és teicoplaninra is. PCR-rel csak a *vanA* gént tudtuk kimutatni, melyek a Tn*1546* transzpozonon találhatók.

Felvetődik a kérdés, ha nem kapnak ezek az állatok glycopeptideket, akkor miért rezisztensek a tápcsatornájukban található *Enterococcus* törzsek? Az izolált, állati eredetű *vanA* pozitív és a humán vonalon előforduló *vanA* gént hordozó törzsek molekuláris szinten történő összehasonlítását végeztük el. Sajnos csak két humán törzset tudtunk a vizsgálatba bevonni, mert hazánkban a *vanA* pozitív humán eredetű törzsek szerencsére nagyon ritkák.

2001-ben és 2002-ben az állati eredetű törzsekben találtunk vancomycin rezisztenciáért felelős gént (*vanA*). 2003-ban és 2004-ben csak olyan mérsékelten vancomycin rezisztens törzseket találtunk, amelyek egyetlen gyakori *van* gént (*vanA*,

vanB, vanC) sem hordoztak. A species szintű meghatározás során nemcsak a leggyakrabban előforduló specieseket tudtuk beazonosítani, hanem az irodalomban is ritkán közölt *vanA* gént hordozó törzset tudtunk azonosítani, ami *Enterococcus mundtii volt*.

A filogenetikai vizsgálatok azt támasztják alá, hogy az állati forrásból származó törzsek túlnyomó része poliklonális eredetű és nincs rokonsági kapcsolatban a humán eredetű törzsekekkel. Azokon az állattenyésztő telepeken ahol az avoparcin betiltása után még öt évvel később is VRE törzseket lehetett izolálni, alapos szűrést lenne célszerű elvégezni a rezisztencia forrás felkutatása érdekében és akár együttműködve az állattartókkal ha a szükség megkívánja akár a tartási technológiát is változtatni lehetne.

10. References

1. Leclercq R, Derlot E, Duval J, Courvalin P. (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med, 319: 157-161.

2. Uttley AH, Collins CH, Naidoo J, George RC. (1988) Vancomycin-resistant enterococci. Lancet, 1: 57-58.

3. Centers for Disease Control and Prevention. (2001) National nosocomial infections surveillance (NNIS) system report, data summary from January 1992–June 2001 issued August 2001. Am J Infect Control, 29: 404–21.

4. Thiercelin ME. (1899) Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogen. C R Soc Biol, 5: 269-271.

5. Sherman JM. (1937) The streptococci. Bacteriol Rev, 1: 3-97.

6. Andrewes FW, Horder TJ. (1906) A study of the streptococci pathogenic for man. Lancet, II: 708-713.

7. Orla-Jensen S. (1919) The lactic acid bacteria. Mem Acad R Soc Danemark Sect Sci Ser, 5: 81-197.

8. Ike Y, Hashimoto H, Clewell DB. (1987) High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. J Clin Microbiol, 25: 1524-1528.

9. Barnes EM. (1956) Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. J Gen Microbiol, 14: 57-68.

10. Deibel RH. (1964) The group D streptococci. Bacteriol Rev, 28: 330-336.

11. Hartman PA,. Reinbold GW,. Saraswat DS. (1966) Indicator organisms - a review. I. Taxonomy of the fecal streptococci. Int J Syst Bacteriol, 16: 197-221.

12. Kalina AP. (1970) The taxonomy and nomenclature of enterococci. Int J Syst Bacteriol, 20: 185-189.

13. Schleifer KH, Kilpper-Balz R. (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Sys Bacteriol, 34: 31–34.

14. Dunne WM Jr,. Dunne BS, Smith. (1996) Watch out where the huskies go. ASM News, 62: 283.

15. Williams AM, Rodrigues UM, Collins MD, (1991) Intrageneric relationships of enterococci as determined by reverse transcriptase sequencing of small subunit rRNA. Res Microb, 142: 67-74.

16. De Graef EM, Devriese LA, Vancanneyt M, Baele M, Collins MD, Lefebvre K, Swings J, Haesebrouck F. (2003) Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus* as a junior synonym of *Enterococcus villorum*. Int J Syst Evol Microbiol, 53: 1069-74.

17. Facklam RR, Carey RB. Streptococci and aerococci. In: Lennette EH, Balows A, Hausler WJ Jr., Shadomy HJ (eds.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C. 1985: 154-175.

18. Devriese, LA, Ceyssens K, Rodrigues UM, Collins MD. (1990) *Enterococcus columbae*, a species from pigeon intestines. FEMS Microbiology Letters, 59: 247-251.

19. Facklam RR, Collins MD. (1989) Identification of enterococcus species isolated from human infection by a conventional test scheme. J Clin Microbial, 27: 731-734.

20. Martinez-Murcia AJ, Collins MD. (1991) *Enterococcus sulfureus*, a new yellow-pigmented *Enterococcus* species. FEMS Microbiol Lett, 64: 69-74.

21. Benno Y, Suzuki K, Suzuki K, Narisawa K, Bruce WR, Mitsuoka T. (1986) Comparison of the fecal microflora in rural Japanese and urban Canadians. Microbiol Immunol, 30: 521-532.

22. Bergan T, Delin C, Johansen S, Kolstad IM, Nord CE, Thorsteinsson SB. (1986) Pharmacokinetics of ciprofloxacin and effect of repeated dosage on salivary and fecal microflora. Antimicrob Agents Chemother, 29: 298-302.

23. Edlund C, Lidbeck A, Kager L, Nord CE. (1987) Comparative effects of enoxacin and norfloxacin on human colonic microflora. Antimicrob Agents Chemother, 31: 1846-1848.

24. Enzensberger R, Shah PM, Knothe H. (1985) Impact of oral ciprofloxacin on the faecal flora of healthy volunteers. Infection, 13: 273-275.

25. Shah PM, Enzensberger R, Glogau 0, Knothe H. (1987) Influence of oral ciprofloxacin or ofloxacin on the fecal flora of healthy volunteers. Am J Med, 2: 333-338.

26. Jett BD, Huycke MM, Gilmore MS. (1994) Virulence of enterococci. Clin Microbiol Rev, 7: 462-478.

27. Hancock LE, Gilmore MS. Pathogenicity of *Enterococci.* In: Fischetti V, Novick R, Ferretti J, Portnoy D, Rood J (eds.) Gram-Positive Pathogens, American Society for Microbiology, Washington DC, 2000: 251-258.

28. Huycke MM, Sahm DF, Gilmore MS. (*1998)* Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerg Infect Dis, 4: 239-249.*

29. Noble CJ. (1978) Carriage of group D streptococci in the human bowel. J Clin Pathol, 31: 1182-1186.

30. Mederski-Samoraj BD, Murray BE. (1983) High-level resistance to gentamicin in clinical isolates of enterococci. J Infect Dis, 147: 751-757.

31. el Amin NA, Jalal S, Wretlind B. (1999) Alterations in *GyrA* and *ParC* associated with fluoroquinolone resistance in *Enterococcus faecium* Antimicrob Agents Chemother, 43: 947-949.

32. Graninger W, Ragette R. (1992) Nosocomial bacteremia due to *Enterococcus faecalis* without endocarditis. Clin Infect Dis, 15:49-57.

33. Livornese LL, Dias S, Samel C, Romanowski B, Taylor S, May P, Pitsakis P, Woods G, Kaye D, Levison ME. (1992) Hospital-acquired infection with vancomycin-resistant Enterococcus faecium transmitted by electronic thermometers. Ann Intern Med, 117: 112-116.

34. Aarestrup FM, Ahrens P, Madsen M, Pallesen LV, Poulsen RL, Westh H. (1996) Glycopeptide susceptibility among Danish *Enterococcus faecium* and *Enterococcus faecalis* isolates of animal and human origin and PCR identification of genes within the VanA cluster. Antimicrob Agents Chemother 40: 1938-1940.

35. Coque TM, Singh KV, Weinstock GM, Murray BE. (1999) Characterization of dihydrofolate reductase genes from trimethoprim-susceptible and trimethoprim-resistant strains of *Enterococcus faecalis*. Antimicrob Agents Chemother, 43: 141-147.

36. Morrison AJ Jr, Wenzel RP. (1986) Nosocomial urinary tract infections due to enterococcus. Ten years' experience at a university hospital. Arch Intern Med, 146: 1549-1551.

37. Felmingham D, Wilson AP, Quintana AI, Gruneberg RN. (1992) *Enterococcus* species in urinary tract infection. Clin Infect Dis, 15: 295-301.

38. Garrison RN, Fry DE, Berberich S, Polk HC. (1982) Enterococcal bacteremia: clinical implications and determinants of death. Ann Surg, 196: 43-47.

39. Wells LD, von Graevenitz A. (1980) Clinical significance of enterococci in blood cultures from adult patients. Infection, 4: 147-151.

40. Jarvis WR, Gaynes RP, Horan TC, Emori TG, Stroud LA, Archibald LK. (1997) CDC NNIS System National Nosocomial Infections Surveillance (NNIS) report, data summary from October 1986-April 1997. AJIC Am J Infect Control 25: 477-487.

41. Wells CL, Jechorek RP, Erlandsen SL. (1990) Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. J Infect Dis, 162: 82-90.

42. Garvey GJ, Neu HC. (1978) Infective endocarditis-an evolving disease Medicine (Baltimore) 57: 105-107.

43. Moellering, RC Jr, Watson BK, Kunz LJ. (1974) Endocarditis due to group D streptococci: comparison of disease caused by *Streptococcus bovis* with that produced by the enterococci. Am J Med 57: 239-250.

44. Scheld WM, Mandell GL. (1984) Enigmatic enterococcal endocarditis. Ann Intern Med, 100: 904-905.

45. Megran DW. (1992) Enterococcal endocarditis. Clin Infect Dis, 15: 63-71.

46. Onderdonk AB, Bartlett JG, Louie T, Sullivan-Seigler N, Gorbach SL. (1976) Microbial synergy in experimental intra-abdominal abscess. Infect Immun, 13: 22-26.

47. Brook I. (1988) Effect of *Streptococcus faecalis* on the growth of *Bacteroides* species and anaerobic cocci in mixed infection. Surgery, 103: 107-110.

48. Hite KE, Locke M, Hesseltine HC. (1949) Synergism in experimental infections with nonsporulating anaerobic bacteria. J Infect Dis, 84: 1-9.

49. Warren RE. (1988) Difficult streptococci. J Hosp Infect, II(Suppl A): 352-357.

50. Clewell DB. (1981) Plasmids, drug resistance, and gene transfer in the genus *Streptococcus.* Microbiol Rev, 45: 409-436.

51. Clewell DB, Gawron-Burke C. (1986) Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu Rev Microbiol, 40: 635-659.

52. Gray JW, Pedler SJ. (1992) Antibiotic-resistant enterococci. J Hosp Infect, 21: 1-14.

53. Murray BE. (1998) Diversity among multidrug-resistant enterococci. Emerg Infect Dis, 4: 37-47.

54. Chenoweth CE, Robinson KA, Schaberg DR. (1990) Efficacy of ampicillin versus trimethoprim-sulfamethoxazole in a mouse model of lethal enterococcal peritonitis. Antimicrob Agents Chemother, 34: 1800-1802.

55. Grayson ML, Thauvin-Eliopoulos C, Eliopoulos GM, Yao JD, DeAngelis DV, Walton L, Woolley JL, Moellering RC Jr. (1990) Failure of trimethoprim-sulfamethoxazole therapy in experimental enterococcal endocarditis. Antimicrob Agents Chemother, 34: 1792-1794.

56. Zervos MJ, Schaberg DR. (1985) Reversal of the in vitro susceptibility of enterococci to trimethoprim-sulfamethoxazole by folinic acid. Antimicrob Agents Chemother, 28: 446-448.

57. Kanematsu E, Deguchi T, Yasuda M, Kawamura T, Nishino Y, Kawada Y. (1998) Alterations in the *GyrA* subunit of DNA gyrase and the *ParC* subunit of DNA topoisomerase IV associated with quinolone resistance in *Enterococcus faecalis.* Antimicrob Agents Chemother, 42: 433-435.

58. Tankovic J, Mahjoubi F, Courvalin P, Duval J, Leclercq R. (1996) Development of fluoroquinolone resistance in *Enterococcus faecalis* and role of mutations in the DNA gyrase *gyrA* gene. Antimicrob Agents Chemother, 40: 2558-2561.

59. Hewitt WL, Seligman SJ, Deigh RA. (1966) Kinetics of the synergism of penicillin-streptomycin and penicillin-kanamycin for enterococci and its relationship to L-phase variants. J Lab Clin Med, 67: 792-807.

60. Moellering RC Jr, Weinberg AN. (1971) Studies on antibiotic syngerism against enterococci. Effect of various antibiotics on the uptake of 14 C-labeled streptomycin by enterococci. J Clin Invest, 50: 2580-2584.

61. Murray BE, Mederski-Samoraj B. (1983) Transferable ß-lactamase: A new mechanism for in vitro penicillin resistance in *Streptococcus faecalis*. J Clin Invest, 72: 1168-1171.

62. Murray BE. (1992) Beta-lactamase-producing enterococci. Antimicrob Agents Chemother, 36: 2355-2359.

63. Fontana R, Ligozzi M, Pittaluga F, Satta G. (1996) Intrinsic penicillin resistance in enterococci. Microb Drug Resist, 2: 209-213.

64. Chen HY. (1986) Resistance of enterococci to antibiotic combinations. J Antimicrob Chemother, 18: 1-4.

65. Eliopoulos GM, Wennersten CB, Cole G, Moellering RC. (1994) In vitro activities of two glycylcyclines against gram-positive bacteria. Antimicrob Agents Chemother, 38: 534-541.

66. Horodniceanu T, Bougueleret L, El-Solh N, Bieth G, Delbos F. (1979) High-level, plasmid-borne resistance to gentamicin in *Streptococcus faecalis subsp zymogenes,* Antimicrob Agents Chemother, 16: 686-689.

67. Ferretti JJ, Gilmore KS, Courvalin P. (1986) Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J Bacteriol, 167: 631-638.

68. Carlier C, Courvalin P. (1990) Emergence of 4',4"-aminoglycoside nucleotidyltransferase in enterococci. Antimicrob Agents Chemother, 34: 1565-1659.

69. Chow JW, Zervos MJ, Lerner SA, Thal LA, Donabedian SM, Jaworski DD, Tsai S, Shaw KJ, Clewell DB. (1997) A novel gentamicin resistance gene in Enterococcus. Antimicrob Agents Chemother, 41: 511-514.

70. Chow JW. (2000) Aminoglycoside resistance in enterococci. Clin Infect Dis, 31: 586-589.

71. Iwen PC, Kelly DM, Linder J, Hinrichs SH, Dominguez EA, Rupp ME, Patil KD. (1997) Change in prevalence and antibiotic resistance of *Enterococcus* species isolated from blood cultures over an 8-year period. Antimicrob Agents Chemother, 41: 494-495.

72. Watanakunakorn C. (1989) The prevalence of high-level aminoglycoside resistance among enterococci isolated from blood cultures during 1980-1988. J Antimicrob Chemother, 24: 63-68.

73. Kirst HA, Thompson DG, Nicas TI. (1998) Historical yearly usage of vancomycin. Antimicrob Agents Chemother, 42: 1303-1304.

74. Wegener HC. (1998) Historical yearly usage of glycopeptides for animals and humans: the American-European paradox revisited. Antimicrob Agents Chemother, 42: 3049.

75. Van Laethem Y, Hermans P, De Wit S, Goosens H, Clumeck N. (1988) Teicoplanin compared with vancomycin in methicillin-resistant *Staphylococcus aureus* infections: preliminary results. J Antimicrob Chemother, 21 Suppl A: 81-87.

76. Marshall CG, Lessard IA, Park I, Wright GD. (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrob Agents Chemother, 42: 2215-2220.

77. Parenti F, Beretta G, Berti M, Arioli V. (1978) Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov sp - Description of the producer strain, fermentation studies and biological properties. J Antibiot, 31: 276-283.

78. Reynolds PE. Inhibitors of bacterial cell wall synthesis. In: Greenwood D, O'Grady (eds.), The Scientific Basis of Antimicrobial Chemotherapy. Cambridge University press, Cambridge, 1985: 13-40.

79. Greenwood D. (1988) Microbiological properties of teicoplanin. J Antimicrob Chemother, 21 Suppl A: 1-13.

80. Neuhaus FC. (1962) The enzymatic synthesis of D-alanyl-D-alanine - Kinetic studies on D-alanyl-D-alanine synthetase. J Biol Chem, 237: 3128-3135.

81. Walsh CT. (1989) Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J Biol Chem, 264: 2393-2396.

82. Arthur M, Reynolds P, Courvalin P. (1996) Glycopeptide resistance in enterococci. Trends Microbiol, 4: 401-407.

83. Woodford N. (1998) Glycopeptide-resistant enterococci: a decade of experience. J Med Microbiol, 47: 849-862.

84. Williamson R, Al-Obeid S, Shlaes JH, Goldstein FW, Shlaes DM. (1989) Inducible resistance to vancomycin in *Enterococcus faecium* D366. J Infect Dis, 159: 1095-1104.

85. Dutka-Malen S, Molinas C, Arthur M, Courvalin P. (1992) Sequence of the *vanC* gene of *Enterococcus gallinarum* BM4174 encoding a D-alanine:D-alanine ligase-related protein necessary for vancomycin resistance. Gene, 112: 53-58.

86. Navarro F, Courvalin P. (1994) Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens.* Antimicrob Agents Chemother, 38: 1788-1793.

87. Fines M, Perichon B, Reynolds P, Sahm DF, Courvalin P. (1999) VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. Antimicrob Agents Chemother, 43: 2161-2164.

88. Perichon B, Reynolds P, Courvalin P. (1997) VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. Antimicrob Agents Chemother, 41: 2016-2018.

89. Quintiliani R Jr, Courvalin P. (1996) Characterization of Tn1547, a composite transposon flanked by the IS*16* and IS*256*-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene, 172: 1-8.

90. Bugg TD, Wright GD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT. (1991) Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry, 30: 10408-10415.

91. Fraimow HS, Jungkind DL, Lander DW, Delso DR, Dean JL. (1994) Urinary tract infection with an *Enterococcus faecalis* isolate that requires vancomycin for growth. Ann Intern Med, 121: 22-26.

92. Woodford N, Johnson AP, Morrison D, Hastings JG, Elliott TS, Worthington A, Stephenson JR, Chin AT, Tolley JL. (1994) Vancomycin-dependent enterococci in the United Kingdom. J Antimicrob Chemother, 33: 1066.

93. Van Bambeke F, Chauvel M, Reynolds PE, Fraimow HS, Courvalin P. (1999) Vancomycin-dependent *Enterococcus faecalis* clinical isolates and revertant mutants. Antimicrob Agents Chemother, 43: 41-47.

94. Farrag N, Eltringham I, Liddy H. (1996) Vancomycin-dependent *Enterococcus faecalis.* Lancet, 348: 1581-1582.

95. Arthur M, Molinas C, Depardieu F, Courvalin P. (1993) Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol, 175: 117-127.

96. Norris AH, Reilly JP, Edelstein PH, Brennan PJ, Schuster MG. (1995) Chloramphenicol for the treatment of vancomycin-resistant enterococcal infections. Clin Infect Dis, 20: 1137-1144.

97. Nicas TI, Zeckel ML, Braun DK. (1997) Beyond vancomycin: new therapies to meet the challenge of glycopeptide resistance. Trends Microbiol, 5: 240-249.

98. Pechere JC. (1996) Streptogramins: A unique class of antibiotics. Drugs, 51 Suppl 1: 13-19.

99. Collins LA, Malanoski GJ, Eliopoulos GM, Wennersten CB, Ferraro MJ, Moellering RC Jr. (1993) In vitro activity of RP59500, an injectable streptogramin antibiotic, against vancomycin-resistant gram-positive organisms. Antimicrob Agents Chemother, 37: 598-601.

100. Chow JW, Donahedian SM, Zervos MJ. (1997) Emergence of increased resistance to quinupristin/dalfopristin during therapy for *Enterococcus faecium* bacteremia. Clin Infect Dis, 24: 90-91.

101. Chow JW, Davidson A, Sanford E, Zervos MJ. (1997) Superinfection with *Enterococcus faecalis* during quinupristin/dalfopristin therapy. Clin Infect Dis, 24: 91-92.

102. Bergeron J, Ammirati M, Danley D, James L, Norcia M, Retsema J, Strick CA, Su WG, Sutcliffe J, Wondrack L. (1996) Glycylcyclines bind to the high-affinity tetracycline ribosomal binding site and evade *Tet(M)*- and *Tet(O)*-mediated ribosomal protection. Antimicrob Agents Chemother, 40: 2226-2228.

103. Someya Y, Yamaguchi A, Sawai T. (1995) A novel glycylcycline, 9-(N,N-dimethylglycylamido)-6-demethyl-6-deoxytetracycline, is neither transported nor recognized by the transposon Tn*10*-encoded metal-tetracycline/H+ antiporter. Antimicrob Agents Chemother, 39: 247-249.

104. Gootz TD, Brighty KE. (1996) Fluoroquinolone antibacterials: SAR mechanism of action, resistance, and clinical aspects. Med Res Rev, 16: 433-486.

105. Cohen MA, Yoder SL, Huband MD, Roland GE, Courtney CL. (1995) In vitro and in vivo activities of clinafloxacin, CI-990 (PD 131112), and PD 138312 versus enterococci. Antimicrob Agents Chemother, 39: 2123-2127.

106. Jones RN, Johnson DM, Erwin ME. (1996) In vitro antimicrobial activities and spectra of U-100592 and U-100766, two novel fluorinated oxazolidinones. Antimicrob Agents Chemother, 40: 720-726.

107. French G, Abdulla Y, Heathcock R, Poston S, Cameron J. (1992) Vancomycin resistance in south London. Lancet, 339: 818-819.

108. Ligozzi M, Lo Cascio G, Fontana R. (1998) *VanA* gene cluster in a vancomycin-resistant clinical isolate of *Bacillus circulans.* Antimicrob Agents Chemother, 42: 2055-2059.

109. Noble WC, Virani Z, Cree RG. (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus.* FEMS Microbiol Lett, 72: 195-198.

110. Hanaki H, Kuwahara-Arai K, Boyle-Vavra S, Daum RS, Labischinski H, Hiramatsu K. (1998) Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. J Antimicrob Chemother, 42: 199-209.

111. Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother, 40: 135-136.

112. Howe RA, Bowker KE, Walsh TR, Feest TG, MacGowan AP. (1998) Vancomycin-resistant *Staphylococcus aureus*. Lancet, 351: 602.

113. Ploy MC, Grelaud C, Martin C, de Lumley L, Denis F. (1998) First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. Lancet 1998 Apr 18;351(9110):1212 No abstract available

114. Murray BE. (1997) Vancomycin-resistant enterococci. Am J Med 102: 284-293.

115. Coque TM, Tomayko JF, Ricke SC, Okhyusen PC, Murray BE. (1996) Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob Agents Chemother, 40: 2605-2609.

116. Schwalbe RS, McIntosh AC, Qaiyumi S, Johnson JA, Morris JG Jr. (1999) Isolation of vancomycin-resistant enterococci from animal feed in USA. Lancet, 353: 722.

117. Bates J, Jordens JZ, Griffiths DT. (1994) Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J Antimicrob Chemother, 34: 507-514.

118. Klare I, Heier H, Claus H, Bohme G, Marin S, Seltmann G, Hakenbeck R, Antanassova V, Witte W. (1995) *Enterococcus faecium* strains with *vanA*-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microb Drug Resist, 1: 265-272.

119. Van der Auwera P, Pensart N, Korten V, Murray BE, Leclercq R. (1996) Influence of oral glycopeptides on the fecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J Infect Dis, 173: 1129-1136.

120. Bates J, Jordens Z, Selkon JB. (1993) Evidence for an animal origin of vancomycin-resistant enterococci. Lancet, 342: 490-491.

121. Swann MM. (1969) Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary under lntensive Livestock Husbandry Systems. HMSO, London.

122. Bonner J. (1997) Hooked on drugs. New Scientist, 153: 24-27.

123. Coates ME, Fuller R, Harrison GF, Lev M, Suffolk SF. (1963) A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br J Nutr, 17: 141-150.

124. Smith WJ. (1993) Antibiotics in feed, with special reference to pigs: a veterinary viewpoint. Anim Feed Sci Tech, 45: 57-64.

125. Aarestrup FM. (1995) Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. Microb Drug Resist, 1: 255-257.

126. Mackinnon JD. (1993) The proper use and benefits of veterinary antimicrobial agents in swine practice. Vet Microbiol, 35: 357-367.

127. Emborg H, Ersboll AK, Heuer OE, Wegener HC. (2001) The effect of discontinuing the use of antimicrobial growth promoters on the productivity in the Danish broiler production. Prev Vet Med, 19: 53-70.

128. Devriese LA , Pot B, Van Damme L, Kersters K, Haesebrouck F. (1995) Identification of enterococcus species isolated from foods of animal origin. Int J Food Microbiol, 26: 187-197.

129. Kaszanyitzky EJ, Tarpai A, Janosi S, Papp M, Skare J, Semjen G. (2002) Development of an antibiotic resistance monitoring system in Hungary. Acta Vet Hung, 50: 189-197.

130. Deasy BM, Rea MC, Fitzgerald GF, Cogan TM, Beresford TP. (2000) A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. Syst Appl Microbiol, 23: 510-522.

131. Jackson CR, Fedorka-Cray PJ, Barrett JB. (2004) Use of a genus- and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol, 42: 3558-3565.

132. Ghidan A, Jeney C, Marodi CL, Csiszar K, Rozgonyi F. (2000) PCR detection of the *vanA* gene in a vancomycin-resistant *Enterococcus faecalis* clinical isolate from Hungary. J Antimicrob Chemother, 46: 325-326.

133. Knausz M, Gartner B, Fomotor I, Ghidan A, Kamotsay K, Rozgonyi F. (2003) Vancomycin-resistant *Enterococcus faecalis* colonization during recovery from *Neisseria meningitidis* cerebrospinal meningitis. Acta Microbiol Immunol Hung 50: 453-457.

134. Dutka-Malen S, Evers S, Courvalin P. (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol, 33: 24-27.

135. Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, Fussing V, Salmenlinna S, Vuopio-Varkila J, El Solh N, Cuny C, Witte W, Tassios PT, Legakis N, van Leeuwen W, van Belkum A, Vindel A, Laconcha I, Garaizar J, Haeggman S, Olsson-Liljequist B, Ransjo U, Coombes G, Cookson B. (2003) Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J Clin Microbiol, 41:1574-1585.

136. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol, 33: 2233-2239.

137. Anonymous. (1984) Water quality - enumeration of faecal enterococci by membrane filtration. ISO 7899/2.

138. Leclerq H, Devriese LA, Mossel DA. (1996) A Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. J Appl Bacteriol, 81: 459-466.

139. Bascomb S, Manafi M. (1998) Use of enzyme tests in characterization and identification of aerobic and facultatively anaerobic gram-positive cocci. Clin Microbiol Rev, 11: 318-340.

140. Knudtson LM, Hartman PA. (1992) Routine procedures for isolation and identification of enterococci and fecal streptococci. Appl Environ Microbiol, 58: 3027-3031.

141. Willey BM, Kreiswirth BN, Simor AE, Faur Y, Patel M, Williams G, Low DE. (1993) Identification and characterization of multiple species of vancomycin-resistant enterococci, including an evaluation of Vitek software version 7.1. J Clin Microbiol, 31: 2777-2779.

142. Devriese LA, Pot B, Collins MD. (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J Appl Bacteriol, 75: 399-408.

143. Flahaud S, Boutibonnes P, Auffray Y. (1997) Les entérocoques dans l'environnement proche de l'homme. Can J Microbiol, 43: 699-708.

144. Manero A, Blanch AR. (1999) Identification of *Enterococcus* spp. with a biochemical key. Appl Environ Microbiol, 65: 4425-4430.

145. Bridge PD, Sneath PH. (1983) A Numerical taxonomy of *Streptococcus.* J Gen Microbiol, 129: 565-597.

146. Chuard C, Reller LB. (1998) Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. J Clin Microbiol, 36: 1135-1136.

147. Fertally SS, Facklam R. (1987) Comparison of physiologic tests used to identify non-beta-hemolytic aerococci, enterococci, and streptococci. J Clin Microbiol, 25: 1845-1850.

148. Singer DA, Jochimsen EM, Gielerak P, Jarvis WR. (1996) Pseudooutbreak of *Enterococcus durans* infections and colonization associated with introduction of an automated identification system software update. J Clin Microbiol 34: 2685-2687.

149. Jensen BJ. (1996) Screening specimens for vancomycin-resistant *Enterococcus.* Lab Med, 27: 53-55.

150. Donabedian S, Chow JW, Shlaes DM, Green M, Zervos MJ. (1995) DNA hybridization and contour-clamped homogeneous electric field electrophoresis for identification of enterococci to species level. J Clin Microbiol, 33: 141-145.

151. Ke D, Picard FJ, Martineau F, Menard C, Roy PH, Ouellette M, Bergeron MG. (1999) Development of a PCR assay for rapid detection of enterococci. J Clin Microbiol, 37: 3497-3503.

152. Facklam RR, Carvalho MGS, Teixeiram LM. (2002) History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In: Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE, Rice LB (eds.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance ASM Press, Washington DC, 1-54.

153. Elliot JA, Facklam RR, Richter CB. (1990) Whole-cell protein patterns of non-hemolytic group B type Ib streptococci isolated from humans, mice, cattle, frogs, and fish. J Clin Microbiol, 28: 628-630.

154. Coque TM, Murray BE. (1995) Identification of *Enterococcus faecalis* strains by DNA hybridization and pulsed-field gel electrophoresis. J Clin Microbiol, 33: 3368-3369.

155. Betzl D, Ludwig W, Schleifer KH. (1990) Identification of lactococci and enterococci by colony hybridization with 23S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol, 56: 2927-2929.

156. Ludwig W, Dorn S, Springer N, Kirchhof G, Schleifer KH. (1994) PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl Environ Microbiol, 60: 3236-3244.

157. McCabe KM, Khan G, Zhang YH, Mason EO, McCabe ERB. (1995) Amplification of bacterial DNA using highly conserved sequences: automated analysis and potential for molecular triage of sepsis. Pediatrics, 95: 165-169.

158. Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW. (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J Clin Microbiol, 34: 818-823.

159. Francis KP, Stewart GS. (1997) Detection and speciation of bacteria through PCR using universal major cold-shock protein primer oligomers. J Ind Microbiol Biotechnol, 19: 286-293.

160. Zolg JW, Philippi-Schulz S. (1994) The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. J Clin Microbiol, 32: 2801-2812.

161. Qamer S, Sandoe JA, Kerr KG. (2003) Use of colony morphology to distinguish different enterococcal strains and species in mixed culture from clinical specimens. J Clin Microbiol, 41: 2644-2646.

162. Borocz K, Szilagyi E, Kurcz A, Libisch B, Glatz K, Gacs M. (2005) First vancomycin-resistant *Enterococcus faecium* outbreak reported in Hungary. Euro Surveill, 10: E050127.

163. Bonora MG, Boldrin C, Bragagnolo L, Cirelli L, De Fatima M, Grossato A, Ligozzi M, Lo Cascio G, Fontana R. (2001) Molecular analysis of *vanA* enterococci isolated from humans and animals in northeastern Italy. Microb Drug Resist, 77: 247-256.

164. Kuhn I, Iversen A, Finn M, Greko C, Burman LG, Blanch AR, Vilanova X, Manero A, Taylor H, Caplin J, Dominguez L, Herrero IA, Moreno MA, Mollby R. (2005) Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. Appl Environ Microbiol, 71: 5383-5390.

165. Yu HS, Seol SY, Cho DT. (2003) Diversity of Tn*1546*-like elements in vancomycin-resistant enterococci isolated from humans and poultry in Korea. J Clin Microbiol, 41: 2641-2643.

166. Lemcke R, Bulte M. (2000) Occurrence of the vancomycin-resistant genes *vanA, vanB, vanCl, vanC2* and *vanC3* in *Enterococcus strains* isolated from poultry and pork. Int J Food Microbiol, 25: 185-194.

167. Prakash VP, Rao SR, Parija SC. (2005) Emergence of unusual species of enterococci causing infections, South India. BMC Infect Dis, 5: 14.

168. Cetinkaya Y, Falk P, Mayhall CG. (2000) Vancomycin-resistant enterococci. Clin Microbiol Rev, 13: 686-707.

169. Arthur M, Courvalin P. (1993) Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob Agents Chemother, 37: 1563-1571.

170. Fraimow HS, Courvalin P. Resistance to glycopeptides in gram-positive pathogens. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI (eds.), Gram-positive pathogens. American Society for Microbiology, Washington DC, 2000: 621-634.

171. Quintiliani R Jr, Courvalin P. (1994) Conjugal transfer of the vancomycin resistance determinant *vanB* between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol Lett, 119: 359-363.

172. Quintiliani R Jr, Courvalin P. (1996) Characterization of Tn*1547*, a composite transposon flanked by the IS*16*16 and IS*256*-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene, 172: 1-8.

173. Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD. (1999) DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. J Clin Microbiol, 37: 1084-1091.

174. Leclercq R, Dutka-Malen S, Duval J, Courvalin P. (1992) Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum*. Antimicrob Agents Chemother, 36: 2005-2008.

175. Depardieu F, Bonora MG, Reynolds PE, Courvalin P. (2003) The *vanG* glycopeptide resistance operon from *Enterococcus faecalis* revisited. Mol Microbiol, 50: 931-948.

176. Balzereit-Scheuerlein F, Stephan R. (2001) Prevalence of colonisation and resistance patterns of vancomycin-resistant enterococci in healthy, non-hospitalised persons in Switzerland. Swiss Med Wkly, 131: 280-282.

177. Huh JY, Lee WG, Lee K, Shin WS, Yoo JH. (2004) Distribution of insertion sequences associated with Tn*1546*-like elements among *Enterococcus faecium* isolates from patients in Korea. J Clin Microbiol, 42: 1897-1902.

178. Perichon B, Casadewall B, Reynolds P, Courvalin P. (2000) Glycopeptide-resistant *Enterococcus faecium* BM4416 is a VanD-type strain with an impaired D-Alanine:D-Alanine ligase. Antimicrob Agents Chemother, 44: 1346-1348.

179. Bustamante W, Alpizar A, Hernandez S, Pacheco A, Vargas N, Herrera ML, Vargas A, Caballero M, Garcia F. (2003) Predominance of *vanA* genotype among vancomycin-resistant Enterococcus isolates from poultry and swine in Costa Rica. Appl Environ Microbiol, 69: 7414-7419.

180. Del Grosso M, Caprioli A, Chinzari P, Fontana MC, Pezzotti G, Manfrin A, Giannatale ED, Goffredo E, Pantosti A. (2000) Detection and characterization of vancomycin-resistant enterococci in farm animals and raw meat products in Italy. Microb Drug Resist, 6: 313-318.

181. Pantosti A, Del Grosso M, Tagliabue S, Macri A, Caprioli A. (1999) Decrease of vancomycin-resistant enterococci in poultry meat after avoparcin ban. Lancet, 354: 741-742.

182. Wegener HC, Aarestrup FM, Jensen LB, Hammerum AM, Bager F. (1998) The association between the use of antimicrobial growth promoters and development of resistance in pathogenic bacteria towards growth promoting and therapeutic antimicrobials. J Anim Feed Sci, 7**:** 7-14.

183. Enright MC, Spratt BG. (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology, 144**:** 3049-3060.

184. Homan W, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, van Embden J, Willems R. (2002) Multilocus sequence typing scheme for *Enterococcus faecium*. J Clin Microbiol 40**:** 1963-1971.

11. LIST OF PUBLICATIONS

Related to the thesis

Papers

1. Ghidan A, Jeney Cs, Marodi CL, Csiszár K, Rozgonyi F. (2000) PCR detection of the *vanA* gene in a vancomycin-resistant *Enterococcus faecalis* clinical isolate from Hungary. J Antimicrob Chemother, 46: 325-326.

2. Kaszanyitzky EJ, Tenk M, Ghidan A, Fehervari GY, Papp M. (2007) Antimicrobial susceptibility of enterococci strains isolated from slaughter animals on the data of Hungarian resistance monitoring system from 2001 to 2004. Int J Food Microbiol, 115: 119-123.

3. Knausz M, Gartner B, Fomotor I, Ghidan A, Kamotsay K, Rozgonyi F. (2003) Vancomycin-resistant *Enterococcus faecalis* colonization during recovery from *Neisseria meningitidis* cerebrospinal meningitis. Acta Microbiol Immunol Hung. 50: 453-457.

4. Rozgonyi F, Ostorhazi E, Marodi CL, Ghidan A. (2001) Resistance to beta-lactams and glycopeptides in staphylococci and streptococci. Acta Microbiol Immunol Hung, 48: 359-391.

5. Ghidan A, Kaszanyitzky EJ, Dobay O, Nagy K, Amyes SGB, Rozgonyi F. (2007) Distribution and genetic relatedness of vancomycin-resistant enterococci (VRE) isolated from healthy slaughtered chicken in Hungary from 2001 to 2004. Acta Vet Hung. (in press)

Citable congress abstracts

1. Rozgonyi F, Knausz M, Fömötör I, Gartner B, Ghidan A. (2000) Vancomycin-resistant *Enterococcus faecalis* superinfection during recovery from *Neisseria meningitidis* cerebrospinal meningitis. Spanish J Chemother, 13, Suppl. 2: 120.

2. Ghidan A, Jeney Cs, Csiszár K, Rozgonyi F. (2000) Detection of vancomycin resistance in *Enterococcus faecalis* using the PCR method. Acta Microbiol Immunol Hung 47/2-3: 219-220.

3. Ghidan A, Csukás Zs, Kamotsay K, Maródi CL, Rozgonyi F. (2001) Antibiotic susceptibility of *Enterococcus* strains isolated from clinical specimens between 1997-2000. Jubilee Meeting of the Hungarian Society for Microbiology, Balatonfüred, October 10-12 2001, Acta Microbiol. Immunol. Hung., 50, 220, 2003.

4. Szabó J, Ghidan A, Dombrádi Zs, Tóth Á, Borbély Á, Miszti C, Andirkó I, Rozgonyi F. (2005) Occurence of vancomycin resistance of enetrococci isolated in Hungarian teaching hospital, Debrecen. Acta Microbiol Immunol Hung, 52: 148.

5. Ghidan A, Dobay O, Kaszanyitzky EJ, Nagy K, Rozgonyi F, Amyes SGB. (2006) Identification and genotyping of vancomycin-resistant enterococci isolated from slaughtered poultry in Hungary from 2001 to 2004. Clin Microbiol Infect, 12: suppl. 4, CD-ROM.

Not related to the thesis

Papers

1. Knausz M, Ghidan A, Grossato A, Rozgonyi F. (2005) Rapid detection of methicillin resistance in teicoplanin-resistant coagulase-negative staphylococci by a penicillin-binding protein 2' latex agglutination method. J Microbiol Methods, 60: 413-416.

2. Dobay O, Rozgonyi F, Ghidan A, Matuz M, Nagy K, Amyes SG. (2006) The first steps towards fluoroquinolone resistance in Hungarian pneumococci. J Chemother. 18: 624-627.

Citable congress abstracts

1. Rozgonyi F, Ghidan A. (1999) A Betadine mikrobiológiája. Sebkezelés–Sebgyógyulás 2: 40.

2. Anderlik P, Antmann K, Ghidan A, Rozgonyi F. (1999) Tüdőkórházban izolált *Staphylococcus aureus* törzsek részletes bakteriológiai vizsgálata. Táplálkozás–Allergia–Diéta 4: 19.

3. Antmann K, Anderlik P, Ghidan A, Rozgonyi F. (2000) Characterization of *Staphylococcus aureus* strains isolated from air and patients during a hospital surveillance. Acta Microbiol Immunol Hung 47/2-3: 219.

4. Rozgonyi F, Ghidan A. (2000) The effect of iodine polyvinylpyrrolodine (Betadine) on multiple antibiotic resistant bacteria and *Candida albicans.* Acta Microbiol Immunol Hung 47/2-3: 211.

5. Knausz M, Ghidan A, Rozgonyi F. (2001) Nosocomial catheter colonization by teicoplanin-resistant coagulase-negative staphylococci in immunocompromised patients. Clin Microb and Infec, 7: Supl. 1: 81-82.

6. Ghidan A, Maródi CL, Csukás Zs, Kamotsay K, Szabó D, Ostorházi E, Rozgonyi F (2002) Incidence of antibiotic resistance in *Staphylococcus aureus* strains in Hungary with special reference to MRSA. Int J Antimicrob Ag, 19, Supl.1: S94.

7. Kristóf K, Kamotsay K, Ghidan A, Rozgonyi F. (2002) Multirezisztens *Staphylococcus epidermidis*ek dominanciája perinatális intenzív centrumok betegeinek hemokultúrás anyagában. Klin Kísérl Lab Med 29/1: 56-57.

8. Kristóf K, Kamotsay K, Ghidan A, Rozgonyi F. (2002) The predominance of multiple resistant *Staphylococcus epidermidis* in bloodstream infections of neonates in neonatal intensive care units. Clin Microb and Infec, 8: Suppl. 1: 182.

9. Kristóf K, Kardos S, Cser V, Ghidan A, Rozgonyi F. (2003) The impact of multiple resistant *S. haemolyticus* in NICU-acquired infection. Antibiotika Monitor, 19: 25.

10. Kristóf K, Szabó Zs, Cser V, Kardos Sz, Ghidan A, Rozgonyi F. (2005) Phenotypical differences in antibiotic resistance between MRSA and MSSA strains isolated from clinical samples of patients treated at the clinics of the Semmelweis University. Clin Microbiol Infect, 11: Suppl. 2: 305.

11. Cekovska Z, Petrovska M, Panovski N, Popovska-Jovanovska K, Ghidan A, Rozgonyi F. (2006) Distribution of MRSA in the Clinical Center Skopje from 2002 to 2005 and prevalence of *mecA* asociated type of resistance in intensive care. Book of Abstracts p. 89.

12. ACKNOWLEDGEMENT

This study was carried out at the Institute of Medical Microbiology, Semmelweis University, Budapest and at the Centre for Infectious Diseases, University of Edinburgh, Edinburgh, Scotland.

First of all I am especially grateful to my supervisors, Professor Sebastian G. B. Amyes and Professor Ferenc Rozgonyi for their continuous indispensable guidance and encourage throughout my PhD, keeping me on the right track and providing the opportunity to work in their laboratories.

Many thanks to Dr. Orsolya Dobay who egged me on to write my thesis and for reading and correcting the grammatical mistakes of the dissertation.

I am grateful to Drs. Éva Kaszanyitzky, Csiszár Károly, Márta Knausz for collection of the enterococcal strains.

Many thanks to Professor Piroska Anderlik for correcting mistakes and for giving good advices.

A special thanks to Natasa Pesti, Imréné Volter and Szilvia Kardos for their valuable technical assistance in the laboratory work and maintenance of the isolates.

Thanks also to Dr. Csaba Jeney, who introduced me in molecular laboratory work in the beginning and taught me for accurate work. Thank you to the mebers of the Centre of Infectious Diseases in Edinburgh for helping me in the molecular studies.

Finally, I would like to thank my family for their support and tolerance during my whole PhD.

This work was financially supported by the British-Hungarian Intergovernmental Science and Technology Cooperation Programme (grant no. GB-30/2003) and the Hungarian National Scientific Research Fund (grant no. OTKA T32473, M36764, T46186).