Glycopeptide-Resistant Enterococci in The Netherlands

Surveillance and Genome analysis

Nicole van den Braak

ISBN 90-73235-92-8

Printed by : Optima Grafische Communicatie, Rotterdam, The Netherlands
Cover design : Martijn La Feber, LFBR, Pernis, The Netherlands

Glycopeptide-Resistant Enterococci in The Netherlands

Surveillance and Genome Analysis

Glycopeptide-Resistente Enterococcen in Nederland

Surveillance en Genoom Analyse

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

woensdag 21 November 2001 om 15.45 uur

door

Nicole Petronella Wilhelmina Cornelia Johanna van den Braak

geboren te Tilburg

PROMOTIECOMMISSIE

Promotor:	Prof. Dr. H.A. Verbrugh
Overige leden:	Prof. Dr. R. de Groot
	Prof. Dr. A.D.M.E. Osterhaus
	Prof. Dr. J.A.A. Hoogkamp-Korstanje
Co-Promotoren:	Dr. H.Ph. Endtz
	Dr. Dr. A.F. van Belkum

Financial supported by: Abott, BaseClear, Bayer, Becton & Dickenson, Bio-Merieux, Merck Sharp & Dome, Oxoid, Pfizer, Pharmacia, Wyeth-LeDerle, Stichting Microbiële Typering

Triple-P , Productschappen vee, vlees en eieren

Contents

Part 3: Molecular analysis of VRE

CHAPTER 1

General Introduction

Nicole van den Braak

The Genus Enterococcus

In 1899, Thiercelin described gram-positive coccoid bacteria isolated from the human intestine and introduced the name "entérocoque" [1]. However, in the beginning of the twentieth century the term *Streptococcus* was more commonly used. In 1937, Sherman developed a new scheme and classified the genus *Streptococcus* into four main groups: pyogenic, viridans and lactic streptococci and enterococci [2]. Enterococci were separated from other *Streptococcus* species as they grow between 10°C and 45°C, in 6.5% NaCl, and at pH 9.6. Moreover, they are able to survive for 30 min. at 60°C and hydrolyze esculine into esculitine. All members of the genus *Enterococcus* react with the Lancefield group D antisera [3]. Recently, DNA hybridization experiments have indicated that enterococci are distinct from streptococci, and subsequently, the genus *Enterococcus* was introduced in 1984 [4]. Biochemical classification divided the genus Enterococcus in 17 different species; *Enterococcus faecium, Enterococcus faecalis, Enterococcus hirae, Enterococcus durans, Enterococcus gallinarum, Enterococcus avium, Enterococcus mundtii, Enterococcus casseliflavus, Enterococcus moledoratus, Enterococcus pseudoavium, Enterococcus solitarus, Enterococcus raffinosus, Enterococcus cecorum, Enterococcus flavescens, Enterococcus columbae, Enterococcus dispar, Enterococcus sacchrolyticus* [5].

Enterococci are part of the human and animal gut-flora, normally 10^5-10^7 CFU can be cultured from one gram of human feces [6], but oral and vaginal colonization has also been described [5]. The number of each of these species found in the human intestine varies with diet and several other factors [7, 8]. *E. faecalis* and *E. faecium* are the species most commonly found in humans. Both species have also been found on plants. *E. faecium* is also part of the intestinal flora of poultry and pigs. *E. durans* is found in human as well as in poultry. In contrast, *E. gallinarum* and *E. avium* appears to be host specific and are mainly found in poultry [5]. The yellow pigmented species *E. casseliflavus* is associated with plants.

Over a period of time, the distribution of organisms involved in nosocomial infections has shifted from Gram-negative to Gram-positive bacteria [9]. Enterococci have emerged as one of the most commonly isolated nosocomial pathogens. Two sources of infections with enterococci have been proposed: first, infections may be caused by enterococcal isolates present in the patient's own

flora; second, infections may be caused by enterococci acquired by transmission in the hospital environment [10]. During the last decade, enterococci have been recognized as one of the leading causes of nosocomial infections. The most prevalent infections caused by enterococci are urinary tract infections (UTI), bacteremia, abdominal wound infections and endocarditis [11, 12]. Most of these infections are caused by *E. faecalis* and only a small number of infections are caused by *E. faecium* [5]. However, in recent years a progressive increase of infections caused by *E. faecium* [13] has been observed. In most cases, it remains difficult to ascertain whether the organism originated from the patients own flora or whether the organism was acquired during hospitalization of the patient.

Antmicrobial Resistance in Enterococci

Antimicrobial resistance in enterococci can be divided in two classes, intrinsic resistance and acquired resistance (table 1). Some bacteria are intrinsically resistant to antimicrobial agents because they either lack the target site for that drug, or the drug is unable to transfer through the organism's cell wall or membrane to reach its site of action. In contrast, acquired resistance is usually transposon or plasmid encoded [5]. From a clinical perspective, multi-resistant enterococci or the vancomycin-resistant enterococci present a major problem. Infections with resistant enterococci are difficult to treat and these organisms show a strong propensity to disseminate and spread from patient to patient in the hospital setting.

Table 1 *Antimicrobial susceptibility of enterococci*

Intrinsic resistance	**Acquired resistance**	
Antimicrobial agent	*Antimicrobial agent*	*Resistance mechanism*
Aminoglycosides (low level)	Aminoglycosides (high-level)	AAC (6'APH(2") enzym
Aztreonam	Ampicillin	*E. faecalis* : β-lactamase
Cephalosporins	Ampicillin	*E. faecium* : PBP5
Clindamycin	Chloramphenicol	cat-encoded enzyme
Imipenem	Erythromycin	ermB-mediated enzyme
Penicillin	Tetracyclines	modification of ribosome protein
Trimethoprim-sulfamethoxazole	Glycopeptides	precusor modification

Adapted from W.Witte Chemotherapy 1999;45:135-145 [5]

Glycopeptides

The emergence of resistance of *Staphylococcus aureus* against penicillin, erythromycin and tetracycline in the mid-1950s, stimulated the development of new antimicrobial agents. In 1954, vancomycin, which belongs to the group of glycopeptide antibiotics, was isolated from *Amycolaptosis orientalis* [14]. Another glycopeptide, teicoplanin, was introduced in 1984 in several European countries [15]. Glycopeptides are relatively large water-soluble molecules that cannot penetrate the lipid outer-membrane of Gram-negative bacteria. In contrast, almost all Gram-positive bacteria are susceptible to the activity of glycopeptides. Glycopeptide antibiotics interact with the terminal D-alanyl-D-alanine group of the pentapeptide side chains of peptidoglycan precursors. Due to this interaction, the cell wall synthesis is inhibited [16].

For 20 years, vancomycin was not used in clinical medicine, because of the frequently observed nefro-toxicity and because of the introduction of penicillinase resistant β-lactam antibiotics. However, in the mid-1980s the interest in vancomycin treatment greatly expanded due to the introduction of a more purified and less toxic formulation of vancomycin and due to a sharp increase in the incidence of infections caused by methicillin resistant and multi-drug resistant staphylococci. From that moment on, vancomycin has remained the treatment of choice for infections with these multi-drug resistant organisms [17] and these circumstances have led to a dramatic increase in the use of vancomycin, especially in the USA. In contrast, the rise of glycopeptide use has been less pronounced in Europe (Figure 1, adapted from reference [18]). In Europe, however, a vancomycin homologue, avoparcin, has been widely used as growth promoter in animal husbandry from its introduction in the late-1970s, until 1997 when it was banned. Large amounts of antibiotics were used in animal husbandry as proved in a recent study from the Health Council of The Netherlands which reported that 40% of the total amount of antibiotics used in the Netherlands was for growth enhancement in animal husbandry in 1997. Approximately 47% were prescribed for prophylaxis and therapy in veterinary medicine, whereas only 13% served human medicinal purposes.

The fight against infections was successful from the mid 1950's. Despite these successes, the development of glycopeptide resistance was noted and the first vancomycin resistant *enterococcus* was isolated in 1986 in France [19].

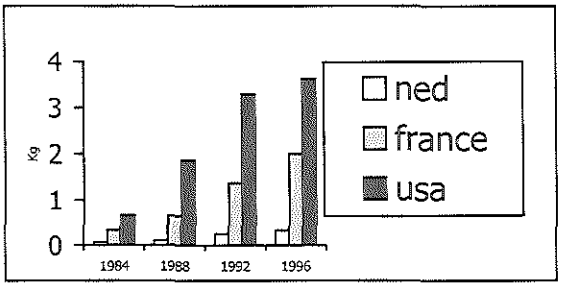


Figure 1: *Intravenous vancomycin use in the USA, France and The Netherlands (NED) (kg/year per 100.000 inhabitants) (adapted from Kirst et al 1998 [18]).*

Mechanisms of Glycopeptide Resistance in Enterococci

Resistance to glycopeptides is due to the synthesis of modified cell wall precursors that show decreased affinity for vancomycin and teicoplanin (Figure 2). The genetics of vancomycin resistance in enterococci has been studied in detail over the past decade and various molecular mechanisms have been elucidated [20-23]. Resistance types can either be intrinsic (low-level resistance to both vancomycin and teicoplanin; e.g. VanC1, VanC2 and VanC3) or acquired (high-level resistance to both vancomycin and teicoplanin [VanA], intermediate level resistance to both glycopeptides [VanD] or variable level of resistance to vancomycin only [VanB]) [24, 25]. A new, VanE-type resistance has recently been described [26]. The VanA/VanB resistances are encoded largely by homologous transposons named *Tn1546* and *Tn1547*, respectively. These transposons are located on self-transferable plasmids and are transferred by conjugation. VanA mediated resistance has been most extensively studied and is associated with the presence of the transposon *Tn1546*. *Tn1546* is a large ± 11-kb transposon and harbors nine genes encoding nine different proteins (Figure 3). These polypeptides can be divided in four functional groups: transposition function (open reading frames 1 and 2 (ORF)), regulation of vancomycin resistance genes (VanR and VanS), resistance to glycopeptides (VanH, VanA and VanX), and synthesis of peptidoglycan (VanY and VanZ). These

two later genes encode accessory proteins that are not essential for the expression of glycopeptide resistance.

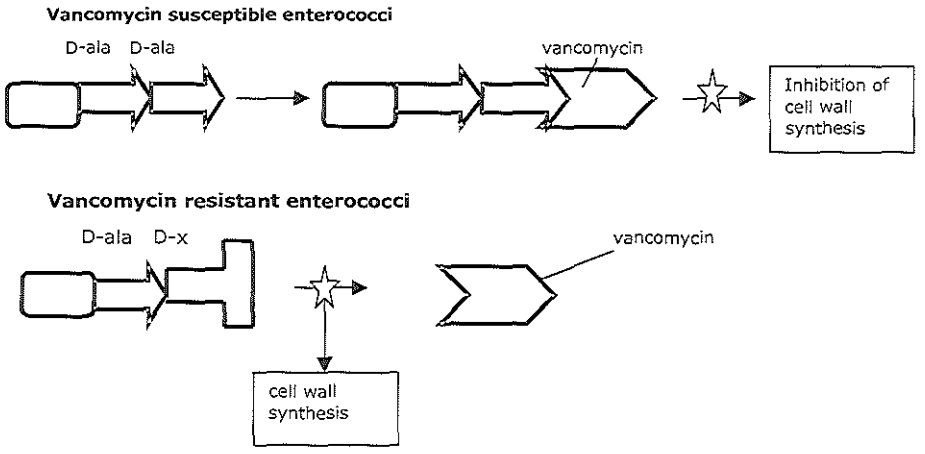


Figure 2: *Vancomycin susceptible enterococci make cell wall precursors (D-ala-D-ala) that have high affinity for vancomycin. After binding of vancomycin to D-ala-D-ala, inhibition of the cell wall synthesis occurs. Vancomycin resistant enterococci synthesize cell wall precursors have low affinity to vancomycin (D-ala-D-lac). Consequently, there will be no irreversible interaction between vancomycin and these precursors and cell wall synthesis continues.*

VanA (ligation D-Lac), *vanH* (dehydrogenase) and *VanX* (removes the terminal D-ala residue) genes are necessary for the synthesis of the depsipeptide D-ala-D-lac, which substitutes for D-ala-D-ala. *VanR* and *vanS* genes regulate the D-ala-D-lac production. The *VanY* and *VanZ* genes encode accessory proteins *that* prevent translocation of D-ala-D-ala precursors to the cell surface (*vanY*). The function of *vanZ* is presently unclear.

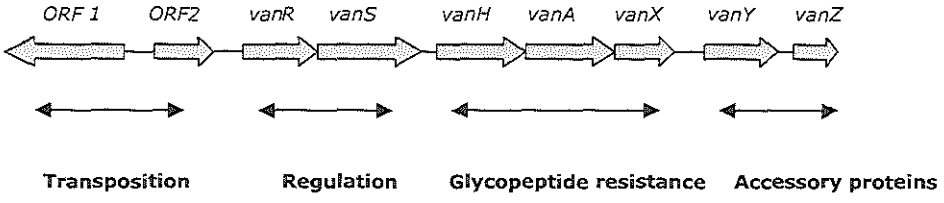


Figure 3: *Map of the Tn1546 transposon in E. faecium BM4147.*

Most of the genes of the *vanB* gene cluster share a large percentage of homology with genes of the *vanA* cluster. One additional gene, *vanW*, is uniquely found in the *vanB* cluster (Figure 4).

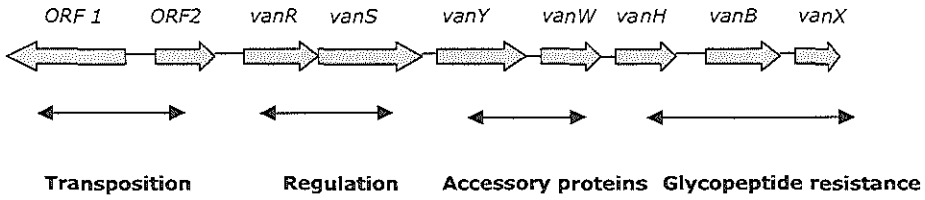


Figure 4: *Map of the Tn1547 transposon in E. faecalis V583.*

VanC mediated intrinsic resistance is characterized by low level resistance and is specific for *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2*) and *E. flavescens* (*vanC3*). Enterococci that carry the *vanC* gene synthesize D-ala-D-serine, which replaced D-ala-D-ala in the precursor molecules. Two newly acquired resistance phenotypes, *vanD* and *vanE* were found in the late nineties in *E. faecium* and *E. faecalis*, respectively [25, 26].

Vancomycin Resistance in other Bacteria

Microbiologists have been forecasting the spread of the *Tn1546* resistance factor to other microbial species as was suggested by in-vitro experiments and in controlled trials in nude mice indicating that this possibility is a realistic one. Conjugative transfer of the *vanA* gene to *Staphylococcus aureus* has been achieved in model studies [27] and has contributed significantly to the discussion on what to do when ultimately vancomycin and methicillin-resistant *S. aureus* (VRSA) is identified in the hospital environment or in a given patient [28]. As a prelude to VRSA, the so-called vancomycin-intermediate *S. aureus* (VISA or GISA) has very recently been isolated from a Japanese patient and two patients in the USA [29, 30]. Fortunately, these strains did not contain the *Tn1546* transposon or other related genes.

In contrast, vancomycin-intermediate and –resistant coagulase-negative staphylococci have been found in larger numbers [31, 32]. For many of these

isolates, interesting mixtures of bacterial phenotypes can be encountered in otherwise genetically homogeneous populations of cells. This type of vancomycin resistance was also not related to one of the *van* genes found in enterococci. In contrast, low-and high level resistances were reported in *Streptococcus* spp., *Arconobacterium* and *Bacillus* spp caused by *vanA* or *vanB* related genes [33].

Prevalence of VRE in the United States versus Europe

Since the first American VRE was isolated in 1987 in Missouri [34], the prevalence of VRE increased enormously and is still on the rise. The National Nosocomial Infection Surveillance (NNIS) system in the USA has revealed a significant increase in the percentage of invasive nosocomial *Enterococcus* strains displaying high-level vancomycin resistance. The figures for the proportion of enterococcal infection due to these so-called vancomycin-resistant enterococci (VRE) have risen in intensive care unit patients from 0.4% in 1989 to 23.4% in 1997. In non-ICU patients the percentage rose from 0.3% to 15.4%, representing a 50-fold increase in a limited time-span. Prevalences of VRE in 1998 and 1999 were published recently (Figure 5) [34]. Most of the outbreaks of VRE in US hospitals are caused by multi-resistant enterococci but in contrast, human VRE colonization outside hospitals and animal derived VRE were not found. It has been suggested that in the USA the large amounts of glycopeptides administered to patients represent the prime determinant driving the development and spread of resistance [35, 36].

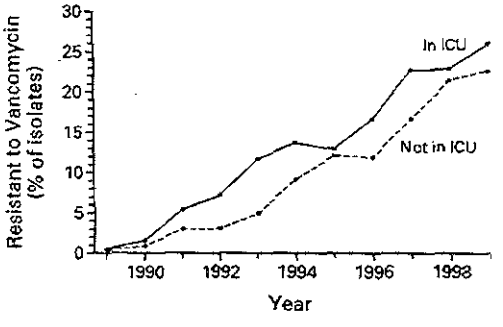


Figure 5: *Resistance of nosocomial isolates of enterococci in the USA (Figure adapted from Barbara Murray (2000), N. Engl. J. Med; 342 (10):710-721 [34].*

Although the very first VRE was reported in France [19], the prevalence of VRE in European hospitals remains low. In contrast to the USA [37], the majority of the hospital-related VRE are genetically heterogeneous. Fortunately, *vanA* related outbreaks of VRE are caused by strains that remained susceptible to other antimicrobial agents. In Europe, VRE have also been detected in non-hospitalized persons. Of major concern is the high prevalence in animals, especially in countries where avoparcin and other antibiotics are commonly used as growth-promoters in animal husbandry. The addition of antibiotics to animal food has been documented as carrying an economical benefit: animals grow faster and attain higher weights [38, 39]. In 1993, the first VRE outside the health care setting was reported in Europe. From that moment VRE were isolated from sources as diverse as sewage in Germany and England, livestock faeces and uncooked-chicken in England and pig and poultry in German. The association between VRE from animal- and human- sources was first described by Bates et al. [40], who reported identical genotypes of VRE in retail poultry cadavers and humans.

Knowing these facts about vancomycin resistant enterococci we became interested in the prevalence and molecular epidemiology of VRE in and outside the hospital setting in the Netherlands.

Outline of the thesis

This thesis describes the detection, prevalence and molecular analysis of vancomycin resistant enterococci in and outside the hospital setting in The Netherlands. The following topics were specifically addressed in this work:

- **Assessment of the quality of commercial assays available for identification of glycopeptide resistance**
  We tested the accuracy of nine different susceptibility test methods for the detection of glycopeptide resistance in enterococci (chapter 2 and 3).
- **Prevalence, risk factors and molecular analysis of vancomycin resistant enterococci in and outside the hospital setting in The Netherlands**
  We determined the prevalence and determinants of VRE carriage in intensive care units and Hematology Oncology wards in nine Dutch hospitals and 200 community based patients between 1995 and 1998 (chapter 4 and 5).
- **Prevalence of VRE in pet-animals**
  We determined the prevalence of VRE in cats and dogs in Rotterdam, The Netherlands (chapter 6b).
- **Prevalence and molecular analysis of VRE in poultry products sold to the public in the Netherlands**
  We determined the prevalence of VRE in poultry product nation wide in order to analyze whether the bacterial flora of consumer poultry serves as gene reservoir (chapter 7).
- **Prevalence of VRE in vegetarians in The Netherlands**
  We described a case-control study in vegetarians versus meat eaters to analyze whether meat can serve a role in dissemination of VRE from animals too human (chapter 6a).
- **Development of molecular techniques to gain more insight in the spread of vancomycin resistant enterococci.**
  We described several molecular techniques to get more insight in to the spread of vancomycin resistant enterococci. The techniques we used were Pulsed Field Gel Electrophoresis, Random Amplification of Polymorphic DNA, transposon analysis using PCR and sequencing, and Amplified Fragment Length Polymorphism. (chapter 8, 9 and 10)

References

1 Thiercelin ME: Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogene. C. R. Soc. Biol (1899) 5:259-271.

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

3 Murray BE: Life and times of the enterococcus. Clin. Microbiol. Rev. (1990) 3:46-65.

4 Schleifer KH, Kilpper-Balz R: 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. Syst. Bacteriol. (1984) 34: 31-34.

5 Witte W, Wirth R, Klare I: Enterococci. Chemother. (1999) 45:135-145.

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

7 Finegold SM, Atteberry HR, Sutter VL: Effect of diet on human faecal flora: comparison of Japanese and American diets. Am. J. Clin. Nutrition. (1974) 27:1456-1469.

8 Hill RJ, Drasar BS, Aries V, Crowther JS, Hawksworth G, Williams REO: Bacteria and etiology of cancer of the large bowl. Lancet (1971) 1:95-100.

9 Weinstein RA, Hayden KH: Multiply drug-resistant pathogens: epidemiology and control. Hosp. Infect. (1998) 4th edition Chapter 15: 215-236.

10 Heck MH, Sham DF, Gilmore MS: Multiple-Drug resistant enterococci: The nature of problem and agenda for the Future. Emerge. Infect. Ids. (1998) 4: 239-249.

11 Emori TG, Gaynes RP: An overview of nosocomial infections, including the role of the clinical microbiology laboratory. Clin. Microbiol. Rev. (1993) 6:428-442.

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

13 Wade JJ: *Enterococcus faecium* in hospitals. Eur. J. Clin. Microbiol. Infect. Dis. (1997) 16:113-119.

14 Leclercq R, Courvalin P: Resistance to glycopeptides in enterococci. Clin. Infect. Dis. (1997) 24:545-556.

15 Trautmann M, Wiedeck H, Ruhnke M, Oethinger M, Marre R: Teicoplanin: 10 years of clinical experience. Infection (1994) 22:430-436.

16 Barna JCJ, Williams DH: The structure and the mode of action of action of glycopeptides antibiotics of the vancomycin group. Annu. Rev. Microbiol. (1984) 38:339-357.

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

18 Kirst HA, Thompson DG, Nicas II: Historical yearly usage of vancomycin. Antimicrob. Agents Chemother. (1998) 42: 1303-304.

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

20 Baptista M, Depardieu F, Courvalin P, Arthur M: Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. Antimicrob Agents Chemother. (1996) 40: 2291-2295.

21 Arthur M, Molinas C, Depardieu F, Courvalin P: Characterization of Tn 1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM 4147. J of Bacteriol (1993) 175: 117-127.

22 Leclercq R, Courvalin P: Resistance to glycopeptides in enterococci. Clinical Infectious Diseases (1997) 24: 545-556.

23 Rao J, Lahiri J, Isaacs L, Weiss RM, Whitesides GM: A trivalent system for vancomycin-D-ala-Dala with higher affinity than avidin biotin. Science (1998) 280:708-711.

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

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

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

27 Noble WC, Virani Z, Cree RGA: Co-transfer of vancomycin and other resistant genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiology Letters (1992) 93: 195-198.

28 Centers for disease control and prevention: interim guides for prevention and control of staphylococcal infections associated with reduced susceptibility to vancomycin. Morbid. Mortal. Weekly Rep. (1997) 46:626-635.

29 Hiramatsu K, Aritaka N, Kawasaki S, Hosodac S, Hori S, Fukuchi Y, Kobayashi I: Dissemination in Japanese hospital of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet (1997) 350:1670-1673.

30 Tenover FC, Lancaster MV, Hill BC, Steward CD, Stocker SA, Hancock CA, O'Hara CM, Clarck NC, Hiramatsu K: Characterization of staphylococci with reduced susceptibility to vancomycin and other glycopeptides. J. Clin. Microbiol. (1998) 36:1020-1027.

31 Van den Braak N, van Belkum A, te Witt R, Verbrugh HA, Endtz HPh: Glycopeptide resistance in Staphylococcus spp. J. Antimicrob. Chemother. (1998) 42:673-675.

32 Sieradzki K, Villary P, Tomasz A: Decreased susceptibility to teicoplanin and vancomycin among coagulase negative methicillin resistant clinical isolates of staphylococci. Antimicrob. Agents Chemother. (1998) 42: 100-107.

33 Kremery Jr V, Sefton A: Vancomycin resistance in Gram-positive bacteria other than *Enterococcus* spp. Int. J. Antimicrob. Agents (2000) 14:99-105.

34 Murray BE: Vancomycin-resistant enterococcal infections. New England J. Med. (2000) 342:710-721

35 Bates J: Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm-animals to human infection. J. Hosp. Infect. (1998) 37:89-101.

36 Luber AD, Jacobs RA, Jordan M, Guglielmo BJ: Relative importance of oral versus intravenous vancomycin exposure in the development of vancomycin resistant enterococci. J. Infect. Dis. (1996) 173:1292-1294.

37 Goossens H: Spread of vancomycin-resistant enterococci: differences between the United States and Europe. Infect. Control. Hosp. Epidemiol. (1998) 19:546-550.

38 Pensack JM, Wang GT, Simkins KL: Avoparcin, a growth promoter promoting feed antibiotic for broiler chicken. Poultry Science (1982) 61:1009-1012.

39 Leeson S, Summers JD, Ferguson AE: Efficiency of avoparcin as a growth promoter for broiler chicken. Can. J. Animal Scien. (1980) 60:275-279.

40 Bates J, Jordens Z, Griffiths DT. 1994. Farm animals as a putative reservoir for vancomycin resistant enterococcal infection in man. J. Antimicrob. Chemother. 34:507-516.

CHAPTER 2

Comparison of Eight Methods, Including the New Vitek GPS-101 Card, to Detect Vancomycin Resistance in Enterococci

Hubert P. Endtz [1], Nicole van den Braak [1], Alex van Belkum [1], Wil H. Goessens [1], Deborah Kreft [1], A. Bernard Stroebel [2], and Henri A. Verbrugh [1].

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands [1]
St Clara Hospital, Rotterdam, The Netherlands [2]

Journal of Clinical Microbiology (1998) 36:592-594

Abstract

A collection of genetically unrelated vancomycin-resistant enterococci (VRE) including 50 *vanA,* 15 *vanB,* 50 *vanC1*, and 30 *vanC2* VRE were used to evaluate the accuracy of eight currently available susceptibility test methods (agar dilution, disk diffusion, E-test, agar screen plate, Vitek GPS-TA and GPS-101, and MicroScan overnight and rapid panels). *VanA* VRE were detected by all methods. *vanB*-VRE were often not detected by Vitek GPS-TA and MicroScan rapid (sensitivity 47% and 53%, resp.), though the new Vitek GPS-101 was found to be a significant improvement. E-test and the agar screen were the only two methods detecting all VRE, including the *vanC1/C2* VRE.

Introduction

The rapid increase in the incidence of infections with vancomycin-resistant enterococci (VRE) in the Western Hemisphere is reason for great concern [1]. The Hospital Infection Control Practices Advisory Committee (HICPAC) recently published recommendations for preventing the spread of vancomycin resistance [2]. An important role is sought for the microbiology laboratories as they, through accurate and timely detection of resistance, are the first line of defense. To date, several studies have been done assessing the accuracy of various antimicrobial susceptibility methods in detecting vancomycin resistance in enterococci [3-9]. Since the occurrence of VRE is increasing in the US [10] and is likely to increase in Europe as well, it is crucial to optimize the laboratory's ability to detect vancomycin resistance. Three different genotypes (*vanA*, *vanB* and *vanD*) have been described that encode for either high-, intermediate-, or low-level acquired glycopeptide resistance, mainly in *Enterococcus faecium* and *Enterococcus faecalis* [11]. In addition, a fourth genotype (*vanC*) has been found in *Enterococcus gallinarum* and *Enterococcus casseliflavus.* This genotype encodes intrinsic, low-level resistance to vancomycin but not to teicoplanin. Antimicrobial susceptibility tests may have problems detecting the low-level glycopeptide resistance phenotype (VanB or VanC). To date, some reports have shown failure of several automated susceptibility test methods to detect vancomycin resistance [8, 9]. In response, the manufacturers of the Vitek system (BioMerieux, Marcy l'Etoile, France) developed a new gram-positive susceptibility card (GPS-101) and updated the software to overcome this problem. Thus, the objective of this study was to evaluate the accuracy of seven currently available
commercial methods, including the Vitek GPS-101 card, to detect VRE compared to a reference agar dilution method [12].

Materials and Methods

A collection of fully characterized VRE strains, representing all the above mentioned genotypes and phenotypes, was used in this study. One hundred and ninety-five enterococci, including 50 *vanA,* 15 *vanB*, 50 *vanC1* VRE (*E.gallinarum)*, and 30 *vanC2* VRE (*E. casseliflavus*) were isolated from patients or poultry products in Europe; the remaining 50 strains lacked these resistance markers and were fully

susceptible to vancomycin. Identification of *Enterococcus* spp. was made on the basis of colonial morphology, pigment production, Gram stain, catalase, pyrrolidonyl arylamidase, and Lancefield group D antigen and by API 32 rapid system. *E. Gallinarum* was identified upon digestion of DNA with SmaI and pulsed-field gel electrophoresis (PFGE) showing all fragments <200kb, and by the presence of the *vanC1* gene [13, 14]. The test strains were carefully selected in order to maximize the variety of resistance genotypes and phenotypes [8]. Identical strains were excluded. All had unique PFGE patterns and were, therefore, genetically unrelated (data not shown). PCR assays for *vanA, vanB, vanC1* and *vanC2* genes were performed as described earlier by Dutka-Malen et al. [15].

Agar dilution and disk diffusion were performed in accordance with the NCCLS guidelines [12, 16] on cation-adjusted Mueller-Hinton (MH) agar (Difco laboratories, Detroit, Mich). E-test (AB Biodisk, Solna, Sweden) was done on MH (Difco) in accordance with the instructions of the manufacturer. The results were read after 24h incubation at 37°C. An agar screen containing 6 µg of vancomycin (BBL Microbiology Systems, Cockeysville, Md) per ml was used as described by Tenover et al. [8] with an inoculum of 10µL (approximately 10^6 CFU) of a 0.5 McFarland standard suspension. The 30-well Vitek GPS-TA, the 45-well Vitek GPS-101 with the updated GUI-software, MicroScan conventional overnight Pos Combo Type 6 panels and MicroScan Rapid Pos Combo Type 1 panels with V.20.30 software (Dade International, West Sacramento, Calif.) were used as recommended by their respective manufacturers. *E. faecalis* ATCC 29212 and *S.aureus* ATCC 29213 were used as quality control strains. The NCCLS breakpoints were used for interpretation of the result [8]. A very major error was defined as an isolate that was resistant by the reference agar dilution method but susceptible with the test method. A major error was defined as an isolate that was susceptible by the reference agar dilution method but resistant with the test method. Thus, lack of sensitivity of a given test was deemed to be more serious clinically than lack of specificity. A minor error was defined as a discrepancy between the results of the reference agar dilution method and the test method that differed only by one interpretation category. However, for the *E. gallinarum* and *E. casseliflavus* strains with MIC 8 to16 µg/ml, both intermediate and resistant results were considered correct, since both interpretation categories correctly distinguish these *vanC1*- or *vanC2*-harboring enterococci from fully susceptible strains (MIC ≤ 4 µg/ml). Similarly, the sensitivity was defined as the ability of the test method to correctly distinguish the *vanA, vanB, vanC1* or *vanC2*-

harboring resistant enterococci from susceptible strains not harboring these genes. Therefore, for strains with intermediate results with the reference agar dilution (MIC, 8 to 16 µg/ml), both intermediate- and resistant-phenotype test results were considered correct.

Results

The MICs of vancomycin with the reference agardilution method are shown by genotype in Table 1. Table 2 presents the percentages of very major, major and minor errors of the different test compared with the reference agar dilution method. The comparative sensitivities of seven methods for the detection of *vanA, vanB, vanC1/C2* VRE are shown in Table 3. All methods were 100% sensitive for the detection of *vanA*-mediated vancomycin resistance. However, it is important to note that for all of the 50 *vanA* VRE MICs of vancomycin was 256 µg/ml, and these strains were therefore detected easily. For *vanB* VRE, the sensitivity dropped to 47, 53 and 93% with Vitek GPS-TA, MicroScan rapid and disk diffusion, respectively. In contrast, Vitek GPS-101, MicroScan conventional, the agar screen and E-test were 100% sensitive for detecting *vanB* VRE. For *vanC1/C2*-VRE, E-test and the agar screen were the only methods that correctly identified all resistant strains as such. High error rates were produced by disk diffusion and by all automated methods (Table 2). The MicroScan conventional panel detected only 7% of the *vanC2 E. casseliflavus.* The sensitivities of the other automated methods varied from 67 to 90% (Table 3). The specificities of the different methods were 96 to 100%.

Table 1: *MH agar determination of MICs for 145 VRE and 50 VSE[a] by genotype*

Organism	No. of isolates for which MIC (µg/ml) was:										
(n)	0.25	0.5	1	2	4	8	16	32	64	128	≥256
VRE											
vanA (50)											50
vanB (15)						1	1	3	1	5	4
vanC1 (50)						30	19	1			
vanC2 (30)						25	4	1			
VSE (50)	1	6	32	9	2						

[a] VSE, vancomycin susceptible enterococci

TABLE 2: *Error rates of seven methods for the detection of vancomycin resistance in enterococci*

	Error rate (%) [a]								
	Very major				Major	Minor			
Method	*vanA* (n=50)	*vanB* (n=15)	*vanC1* (n=50)	*vanC2* (n=30)	- (n=50)	*vanA* (n=50)	*vanB* (n=15)	*vanC1* (n=50)	*vanC2* (n=30)
E-test	0	0	0	0	0	0	13	2	0
Disk diffusion	0	0	0	0	2	0	27	50	37
Agar screen	0	0	0	0	4	-	-	-	-
Microscan									
Conventional	0	0	0	3	0	0	0	24	90
Rapid	0	33	0	0	0	0	27	14	10
Vitek									
GPS-TA	0	40	0	0	4	0	13	28	37
GPS-101	0	0	0	0	4	2	7	12	30

[a] Relative to the NCCLS reference agar dilution assay. Error types are defined in the text.

TABLE 3: *Sensitivities of seven methods for the detection of vanA, vanB, and vanC1/C2-enterococci*[a]

	sensitivity (%) for VRE			
method	*VanA* (n=50)	*VanB* (n=15)	*VanC1* (n=50)	*VanC2* (n=30)
E-test	100	100	100	100
Disk diffusion	100	93	52	63
Agar screen	100	100	100	100
Microscan				
Conventional	100	100	76	7
Rapid	100	53	86	90
Vitek				
GPS-TA	100	47	72	67
GPS-101	100	100	88	73

[a] For *vanB, vanC1* and *vanC2* strains with MICs of 8 to16 µg/ml, both intermediate- and resistant-phenotypes were considered correct.

Discussion

Earlier studies have reported on the performance of commercial and reference methods for the detection of vancomycin resistance in enterococci [3-10]. Surprisingly, none of these studies were performed in Europe. Some of the studies report on the difficulties of automated methods in detecting low-level or intermediate-level vancomycin-resistance [8, 9]. In the study by Tenover et al., the performance of the MicroScan rapid panel and the Vitek GPS-TA card were problematic, with very major error rate of 20.7 and 10.3%, respectively. Many errors occurred with *E. casseliflavus, E. gallinarum* and *vanB* VRE. We confirm the failure of these two methods. The MicroScan rapid and Vitek GPS-TA produced 33 and 40% very major errors with *vanB*-strains, respectively (Table 2). However, no very major errors occurred with MicroScan conventional or with Vitek GPS-101. No susceptible (vancomycin MIC ≤ 4 µg/l) *E. gallinarum* or *E. casseliflavus* was found, possibly due to the fact that the strains were initially isolated with the use of a selective broth medium containing 6 µg of vancomycin per liter. Since for 78 of the 80 *E. gallinarum* and *E. casseliflavus* had vancomycin MICs were in the intermediate category (MIC 8 to16 µg/ml), most errors in these species were, by definition, minor errors. For one *vanC1 E. gallinarum* and one *vanC2 E. casseliflavus*, the MIC of vancomycin was 32 µg/ml. The latter was incorrectly reported as susceptible by MicroScan conventional panel, and this result was scored as very major error (Table 2). The MicroScan conventional panel and MicroScan rapid had 24 and 14% minor errors, respectively, with *vanC1 E. gallinarum* but 90 and 10%, respectively, with *vanC2 E. casseliflavus.* Vitek GPS-TA and Vitek GPS-101 produced 28 and 12% minor errors, respectively, with *vanC1 E. gallinarum* and 37 and 30%, respectively, with *vanC2 E. casseliflavus*. The minor error rates of the disk diffusion in *E. gallinarum* and *E. casseliflavus* were 50 and 37%, respectively. Swenson et al. reported minor error rates of 14,5% of total values. However, their collection of 100 VRE included only 10 *E. gallinarum* or *E. casseliflavus* isolates, and the most significant errors in detection were in fact made mainly with these strains [5]. E-test and the agar screen were the only methods that correctly detected all VRE in our study. Light growth was observed on the agar screen with two vancomycin-susceptible strains with (MIC, 4 µg/ml). This high sensitivity is in concordance with recent data reported by Willey et al. [9]. They found the agar screen plate (using the same vancomycin concentration as used in our study) to be 100% sensitive and

specific. In another study, which included only a small number of strains with MICs in the 8-16 µg/ml range, E-test proved to be a reliable method compared to agar dilution [4]. The prevalence and the clinical relevance of *E. casseliflavus* and *E. gallinarum* remain to be elucidated. These VRE are often misidentified by commercial identification systems (data not shown [3], and their intermediate level of resistance may not be detected. It is likely that these two species are being underreported in the literature [10, 17].

In conclusion, *vanA* VRE are detected by all methods. *VanB* VRE are often not detected by Vitek GPS-TA and MicroScan rapid panel, though the new Vitek GPS-101 appears to be a significant improvement. All methods except E-test and the agar screen continue to show problems in the detection of *vanC1/C2* VRE. The agar screen appears to be the most reliable and easy to perform method for routine screening, if detection of *vanA-, vanB-, and vanC1/C2*-mediated resistance in enterococci is required. The new 45-well Vitek GPS-101 shows improved sensitivity, compared to the Vitek GPS-TA without significant loss of specificity.

Acknowledgments

We gratefully acknowledge Marian Humphrey for reading the English version of the manuscript.

References

1. Murray BE : Editorial response: What can we do about vancomycin-resistant enterococci. Clin. Infect. Dis. (1995) 20:1134-1136.
2. Hospital Infection Control Practices Advisory Commitee: Recommendation for preventing the spread of vancomycin resistance: Recommendation of the Hospital Infection Control Practices Advisory Committee (HICPAC). Am. J. Infect. Control (1995) 23:87-94.
3. Sahm DF, Olsen L : In vitro detection of enterococcal vancomycin resistance. Antimicrob. Agents Chemother. (1990) 34:1846-1848.
4. Schulz JE, Sahm DF : Reliability of the E test for detection of ampicillin, vancomycin, and high-level aminoglycoside resistance to *Enterococcus* spp. J. Clin. Microbiol. (1993) 31:3336-3339.
5. Swenson JM, Hill BC, Thornsberry C: Problems with the disk diffusion test for detection of vancomycin resistance in enterococci. J. Clin. Microbiol. (1989) 27: 2140-2142.
6. Swenson JM, Ferraro MJ, Sahm DF, Charache P, The National Committee For Clinical Laboratory Standards Working Group On Enterococci, Tenover FC: New vancomycin disk diffusion breakpoints for enterococci. J. Clin. Microbiol. (1992) 30:2525-2528.
7. Tenover FC, Tokars J, Swenson JM, Paul S, Spitalny K, Jarvis W : Ability of laboratories to detect antimicrobial agent-resistant enterococci. J. Clin. Microbiol. (1993) 32: 1700-1704.
8. Tenover FC, Swenson JM, O'Hara CM, Stocker SA: Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci. J. Clin. Microbiol. (1995) 33:1524-1527.

9. Willey BM, Kreiswirth BN, Simor AE, Williams G, Scriver GR, Phillips A, Low DE: Detection of vancomycin resistance in *Enterococcus* species. J. Clin. Microbiol. (1992) 30:1621-1624.
10. Centers for Diseases Control and Prevention: Nosocomial enterococci resistant to vancomycin-United States, 1889-1993. Morbid. Mortal. Weekly Rep. 42:597-599.
11. Leclercq R, Courvalin P : Resistance to glycopeptides in enterococci. Clin. Infect. Dis. (1997) 24:545-556.
12. National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. (1997) 4th ed. Approved standards M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa.
13. Endtz H Ph, Van den Braak N, Van Belkum A, Kluytmans JAWJ, Koeleman JGM, Spanjaard L, Voss A, Weersink AJL, VandenBroucke-Grauls CMJE, Buiting AGM, Van Duin A, Verbrugh HA: Fecal carriage of vancomycin-resistant enterococci in hospital- and community-based patients in The Netherlands. J. Clin. Microbiol (1997) 35:3026-3030.
14. Leclerq R, Dutka-Malen S, Duval J, Courvalin P: Vancomycin resistance gene *vanC* is specific for *Enterococcus gallinarum*. Antimicrob. Agents Chemother. (1992) 36:200-2008.
15. Dutka-Malen S, Evers S, Courvalin P: Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. (1995) 33:24-27.
16. National Committee for Clinical Laboratory Standards: Performance standards for antimicrobial disk susceptibility tests, (1997) 6th ed. Approved standards M2-A6. National Committee for Clinical Laboratory Standards, Villanova, Pa.
17. Michel M, Gutmann L: Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci: therapeutic realities and possibilities. Lancet (1997) 349:1901-1906.

CHAPTER 3

Accuracy of the VITEK®2 System to Detect Glycopeptide-Resistance in Enterococci

Nicole van den Braak, Wil Goessens, Alex van Belkum, Henri A. Verbrugh and Hubert Ph. Endtz

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands

Journal of Clinical Microbiology (2001) 39:351-353

Abstract

We evaluated the accuracy of the VITEK®2 fully automated system to detect and to identify glycopeptide-resistant enterococci (GRE) compared to a reference agar dilution method. The sensitivity of vancomycin susceptibility testing with VITEK®2 for the detection of *vanA*-, *vanB* and *vanC1*-strains was 100%. The sensitivity of vancomycin susceptibility testing of *VanC2* strains was 77%. The sensitivity of teicoplanin susceptibility testing of *vanA* strains was 90%. Of 80 *VanC* enterococci, 78 (98%) were correctly identified by VITEK®2 as *Enterococcus gallinarum/Enterococcus casseliflavus.* Since the identification and susceptibility data are produced within 3 h and 8 h, respectively, VITEK®2 appears a fast and reliable method for detection of GRE in microbiology laboratories.

Introduction

The prevalence of glycopeptide resistance among clinical isolates of *Enterococcus* spp., first described in 1986 [1], is ever increasing, thereby limiting the treatment options for infections caused by glycopeptide-resistant enterococci (GRE). Molecular epidemiology has elucidated several determinants of glycopeptide resistance as well as gene reservoirs and has increased our awareness of the spread of GRE in hospitals and in the community [2-4]. However, although the microbiology laboratories have been delineated as the first line of defense to control the spread of GRE within our hospitals [5], many technical problems concerning the laboratory detection of GRE still exist. Previous studies have reported on problems with the detection of *vanB-, vanC1-* and *vanC2*-type strains, in particular [6-8]. Both convential and automated methods have problems in detecting these particular genotypes. The manufacturers of commercial susceptibility testing methods have joined in their efforts to contain the problem of increasing resistance, by developing new and rapid susceptibility test methods. Our main objective in this study was to evaluate the ability of VITEK®2 to determine vancomycin and teicoplanin resistance in strains containing *vanA, vanB, vanC1* or *vanC2* genes. The performance of VITEK GPI and VITEK®2 for the identification of *E. faecalis* and *E. faecium* has been evaluated by others [9, 10]. Therefore, our second objective was to evaluate the performance of VITEK®2 for the identification of *vanC* enterococci up to the species level, as most automated methods have problems with the identification of *Enterococcus gallinarum* and *Enterococcus casseliflavus* [6-8].

Materials and Methods

Bacterial strains

A collection of genetically distinct GRE and glycopeptide susceptible enterococci (GSE) from diverse sources was used in this study. This collection was assembled and characterized by molecular methods in a previous study [6]. A total of 195 enterococci, including *vanA* (n=50), *vanB* (n=15) *vanC1* (n=50),

vanC2 (n=30) and GSE (n=50), were isolated from patients, pets or poultry products in The Netherlands.

Identification

All enterococci were identified to the species level on the basis of colony morphology, Gram stain, pyrase and catalase testing, pigment production, the presence of the Lancefield Group D antigen and Rapid ID32 Strep (bioMérieux, 's Hertogenbosch, the Netherlands). PCR assays for *vanA, vanB, vanC1* and *vanC2* genes [11] were used to assess the presence of the various glycopeptide resistance genes. Strains carrying the *vanC1* or *vanC2* genes were identified as *E. gallinarum* and *E. casseliflavus*, respectively. The identification of *E. gallinarum* was confirmed by pulsed-field gel electrophoresis (PFGE) after digestion with *SmaI,* which led to the display of macrorestriction fragments of less then 200 kb only [3]. All strains were genetically characterized by PFGE, and only unique strains were included in the study.

Susceptibility testing

Susceptibility results for vancomycin and teicoplanin obtained by agar dilution performed according to the guidelines of the NCCLS [12] were used as a reference method. The VITEK®2 system was used according to the instructions of the manufacturer (bioMerieux, Marcy l' Etoile, France); ID-Gram Positive Cocci (GPC) cards were used for identification. The Antimicrobial Susceptibility Testing (ASP) P516 card was used for susceptibility testing. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. MICs were interpreted as indicating susceptible, intermediate or resistant categories according to the breakpoints recommended by the NCCLS. A very major error was defined as occurring when an isolate that was resistant by the agar dilution method appeared to be susceptible by the test method. A major error was defined as occurring when an isolate that was susceptible by the reference agar dilution method was scored resistant by the test method. Thus, lack of sensitivity of a given test was considered to be a more serious handicap than lack of specificity. A minor error was defined as a discrepancy between the results of the reference agar dilution method and the test method that differed only by one interpretation category. The sensitivity was defined as the ability of the test method to correctly distinguish the *vanA, vanB, vanC1* or *vanC2*-

harbouring resistant enterococci from susceptible strains not harboring these genes. However, for enterococcal strains with vancomycin MICs of 8-16 µg/ml both intermediate and resistant results were considered correct, since both interpretation categories correctly distinguish these enterococci from fully susceptible strains (vancomycin MIC ≤ 4 µg/ml; teicoplanin MIC ≤ 8 µg/ml). For reporting the accuracy of VITEK®2 compared to the reference method, overall agreement was defined as 100X (number of strains with the reference MIC ± 1 dilution/total number of strains tested).

Results

Table 1 shows the susceptibility results for the 195 enterococci obtained by VITEK®2 and the reference agar dilution method for vancomycin and teicoplanin. The sensitivity of vancomycin susceptibility testing with VITEK®2 for the detecting of *vanA*-, *vanB* and *vanC1*-strains was 100%. However, it is important to note that vancomycin MICs for all *vanA* strains in this study were >256 µg/ml. The sensitivity of the system for detecting *vanC2* strains was 77%. Several minor errors were found in the *vanB, vanC1, vanC2* enterococci as well as in GSE group: 1% (2 of 195), 5.6% (11 of 195), 4.1% (8 of 195) and 0.5% (1 of 195), respectively. No major- or very major errors were encountered in the GRE or GSE group.

In contrast, 3 minor errors in 50 samples (6%) and 5 very major errors in 50 samples (10%) occurred when the teicoplanin susceptibility test results were analyzed for detecting *vanA* strains. These 5 very major errors were confirmed several times, both by bioMerieux researchers and in our laboratory. However, the teicoplanin MICs of these 5 strains as determined by re-testing with VITEK®2 ranged from 4 to >32 mg/L on different testing days. Neither the isolation media used nor the inocula can explain these major errors (data not shown). All *vanB*, *vanC1* and *vanC2* enterococci susceptible for teicoplanin were classified correct with the VITEK®2 system.

The overall agreement of vancomycin susceptibility testing with the VITEK®2 system compared with the reference agar dilution method was 94% (184 of 195) (Table 2); the overall agreement of teicoplanin testing results between the two methods was 97% (189 of 195).

Table 1: *Determination using VITEK®2 automated system versus standard agar dilution of vancomycin and teicoplanin MICs of 145 GRE and 50 GSE by genotype*

		No. of isolates with the following MIC (mg/L)											
		vancomycin						teicoplanin					
Organism (no.)		<=1	2	4	8	16	>= 32	<=1	2	4	8	16	>= 32
vanA-GRE (50)	V2						50				5	3	42
	AD						50						50
vanB-GRE (15)	V2						15	14			1		
	AD				1	1	13	15					
vanC1-GRE (50)	V2				21	19	10	50					
	AD				30	19	1	50					
vanC2-GRE (30)	V2			1	6	23		30					
	AD				25	4	1	30					
GSE (50)	V2	42	6	1	1			49	1				
	AD	39	9	2				50					

Table 2: *Comparison of vancomycin MICs determined by VITEK®2 with MICS determined by the reference agar dilution method for 195 isolates of Enterococcus spp.*

	No. of Vitek2 MICs of vancomycin within indicated log of reference MIC							
Organism (no.)	>-2	-2	-1	0	+1	+2	>+2	Agreement (%)*
vanA-GRE (50)					50			100
vanB- GRE (15)					13	1	1	93
vanC1-GRE (50)				8	22	14	6	88
vanC2-GRE (30)			2	10	18			93
GSE (50)				6	40	4		100
Total		2	2	24	143	19	7	95

* agreement % = number of strains with reference MIC ± one dilution divided by the total number of strains x 100%

Of 80 *vanC* enterococci, 78 (98%) were classified by VITEK®2 as *E. gallinarum/E. casseliflavus* and 2 were classified as unidentified by VITEK®2. This is a significant improvement over other non-automated methods. Although VITEK®2 separates the vanC1/vanC2 enterococci from the other enterococci it can not differentiate between *E. gallinarum* and *E. casseliflavus*. However, the clinical significance of separating these two species is doubtful.

The mean time for obtaining antimicrobial susceptibility results for the enterococci tested in this study was 8 h and 6 min (range, 5h 25 min to 14 h 30 min). All identification data were obtained within 3 h after starting the identification procedure, as guaranteed by the manufacturer.

Discussion

Several studies have reported the accuracy of automated methods to detect GRE. Most of these studies identified major problems in detection of enterococci harboring the *vanB, vanC1* and *vanC2* genes [6-8]. We previously reported very major errors, which occurred with the Vitek GPS-TA card. However, most strains were correctly classified with the new VITEK GPS-101 card. The VITEK GPS-101 card had a sensitivity of 100% detecting VanB phenotypes [6]. In this study no problems were found to detect *vanB* strains. Minor errors (n=22) occur with the VITEK®2 system in detecting GRE. However, the 2 minor errors in the *vanB* group and 10 minor errors in *vanC1* enterococci were intermediate strains reported as resistant. VITEK®2 is the first automated susceptibility method that tests vancomycin as well as teicoplanin for antimicrobial susceptibility, which is important for the description of the resistance phenotype.

For identification and susceptibility testing, most conventional methods require a full 24 h of incubation, VITEK®2 reports susceptibility results in approximately 8 h. Barenfanger et al. [13] have demonstrated that rapid reporting of identification and susceptibility results may have important benefits in terms of patient outcome and cost effectiveness. Moreover, Doern et al., reported that rapid identification and susceptibility tests results even reduced morbidity and mortality [14]. VITEK®2 provides enterococcal susceptibility data in approximately 8 h. Although this is significant faster than overnight convential methods, it implies that results can still not be obtained in one working shift. To maximize the impact of rapid testing, further improvement of the speed without

compromising the accuracy of the test method is desired. In the mean time, we have experienced that prolonging the opening hours of the microbiology laboratory and adapting the workflow in order to proceed to earlier reports is an achievable goal.

In conclusion, The VITEK®2 appears to be an improvement over convential methods for the detection of vancomycin resistance in enterococci. However, detection of teicoplanin resistance in enterococci containing the *VanA* gene needs to be reassessed. Although the detection time was reduced to 8 h, further improvement of the algorithm and further reduction of the detection time may considerably increase the impact of rapid testing on patient care [1,3].

References

1. Leclerq R, Derlot E, Duval J: Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. New Engl. J. Med. (1988) 319:157-161.
2. Centers for Disease Control and Prevention: Nosocomial enterococci resistant to vancomycin-United States, 1989-1993. Morbid. Mortal. Weekly Rep. (1993) 42:597-599.
3. Endtz HPh, Van den Braak N, Van Belkum A, Kluytmans AJW, Koeleman JGM, Spanjaard L, Voss A, Weersink AJL, Vandenbroucke-Grauls CMJE, Buiting AGM, Van Duin A, Verbrugh HA: Fecal carriage of vancomycin-resistant enterococci in hospital- and community- based patients in the Netherlands. J. Clin. Microbiol. (1997) 35:3026-3030.
4. Goossens H: Spread of vancomycin-resistant enterococci: differences between the United States and Europe. Infect. Control Hosp. Epidemiol. (1998) 19:546-550.
5. HICPAC: Recommendation for preventing the spread of vancomycin resistance: Recommendation of the Hospital Infection Control Practices Advisory Committee (HICPAC). Am. J. Infect. Control (1995) 23:87-94.
6. Endtz HPh, Van den Braak N, Van Belkum A, Goessens WH, Kreft D, Stroebel AB, Verbrugh HA: Comparison of eight methods to detect vancomycin resistance in enterococci. J. Clin. Microbiol. (1995) 36:592-594.
7. Kohner PC, Patel R, Uhl JR, Garin KM, Hopkins MK, Wegener LT, Cockerill III FR: Comparison of agar dilution, broth microdilution, E-test, disk diffusion, and automated Vitek methods for testing susceptibility of enterococcus spp. to vancomycin. J. Clin. Microbiol. (1997) 35:3258-3263.
8. Tenover FC, Swenson JM, Ohara SM, Stocker SA. Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci. J. Clin. Microbiol. (1995) 33:1524-1527.
9. Garcia-Garotte F, Cercenado E, Bouza E: Evaluation of a new system, VITEK2, for identification and antimicrobial susceptibility testing of enterococci. J. Clin. Microbiol. (2000) 38:2108-2111.
10. Liassine N, Frei R, Jan I, Auckenthaler R: Characterization of Glycopeptide-Resistant Enterococci from a Swiss Hospital. J. Clin. Microbiol. (1998) 36:1853-1858.
11. Dutka-Malen S, Evers S, Courvalin P: Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. (1995) 33:24-27.
12. National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 4th ed. Approved standards M7-A5. National Committee for Clinical Laboratory Standards, Villanova, Pa.2000.

13. Barenfanger J, Drake C, Kacich G: Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. J. Clin. Microbiol. (1999) 37:1415-1418.
14. Doern GV, Vautour R, Gaudet M, Levy B: Clinical impact of rapid in vitro susceptibility testing and bacterial identification. J. Clin. Microbiol. (1994) 32:1757-1762.

CHAPTER 4

Fecal Carriage of Vancomycin-Resistant Enterococci in Hospital- and Community-Based Patients in The Netherlands

Hubert Ph. Endtz[1], Nicole van den Braak[1], Alex van Belkum[1], Jan A.J.W. Kluytmans[2, 3], Hans G.M. Koeleman[2, 4], Lodewijk Spanjaard[2, 5], Andreas Voss[2, 6], Annemarie J.L. Weersink[2, 7], Christina M.J.E. Vandenbroucke-Grauls[2, 4], Anton G.M. Buiting[8], Anneriet van Duin[9], and Henri A. Verbrugh[1, 2]

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands[1] ; Working party Hospital infection Epidemiology Netherlands (WHEN) [2].
Ignatius Hospital, Breda[3]; Free University Hospital, Amsterdam[4]; Academic Medical Center, Amsterdam[5]; Radboud University Hospital, Nijmegen[6]; University Hospital, Utrecht[7]; Elisabeth Hospital, Tilburg [8]; Zuiderziekenhuis, Rotterdam[9]

Journal of Clinical Microbiology (1997) 35:3026-3031

Abstract

In order to determine the prevalence of vancomycin-resistant enterococci (VRE) in The Netherlands, 624 hospitalized patients from ICU or hemato-oncology wards in nine hospitals and 200 community-based patients were screened for VRE colonization. In 49% of the hospitalized patients and in 80% of the community-based patients, enterococci were found. Of these strains 43% and 32%, respectively, were *Enterococcus faecium*. VRE were isolated in 12/624 (2%) and 4/200 (2%), respectively. PCR analysis of these 16 strains and 11 additional clinical VRE isolates from one of the participating hospitals revealed 24 *VanA*-, 1 *VanB*-, and 2 *VanC1*-gene containing strains. All strains were cross resistant to avoparcin, but sensitive to the novel glycopeptide antibiotic LY333328. Genotyping of the strains with arbitrarily primed PCR and pulsed-field gel electrophoresis revealed a high degree of genetic heterogeneity. This underscores a lack of hospital-driven endemicity of VRE-clones. It is suggested that the VRE in hospitalized patients have originated from presently unknown sources in the community and may be linked to the extensive use of the glycopeptide avoparcin as growth promoting agent in animals.

Introduction

Enterococcus spp. have recently emerged as important nosocomial pathogens [1]. According to the data from the National Nosocomial Infections Surveillance System, enterococci are the fourth leading cause of nosocomial infections in the United States [2]. Enterococcal infections that have frequently been reported include urinary tract infections, bacteremia, endocarditis, intra-abdominal infections and surgical wound infections [3]. *E. faecalis* is commonly isolated from the human gastro-intestinal tract, whereas *E. faecium* is less frequent [4]. This latter species, however, is noted for its antimicrobial resistance. Vancomycin-resistant *E. faecium* (VREF) have emerged in a setting of increasing high-level resistance of enterococci against penicillins and aminoglycosides [5]. During the last years, nosocomial outbreaks due to VREF have been described [6,7]. The emergence of VREF has raised serious concerns [5] and in response, the Hospital Infections Control Practices Advisory Committee (HICPAC) in collaboration with the Centers for Disease Control and Prevention (CDC) has developed recommendations to prevent the spread of vancomycin-resistant enterococci (VRE) [8]. Given the concern that vancomycin-resistance genes may transfer from enterococci to *Staphylococcus aureus*, a phenomenon that has been observed *in vitro* [4], control measures have already been proposed, should vancomycin-resistant *S. aureus* eventually arise [9].

The microbiology laboratory has an important role in the detection, reporting and control of VRE. The HICPAC document emphasizes the need for routine susceptibility testing of all enterococci isolated from clinical specimens. Furthermore, in hospitals where VRE have not yet been detected, periodic culture surveys of stools or rectal swabs of patients at high risk for VRE infection or colonization is indicated [8]. In The Netherlands, no systematic study has been done to evaluate the prevalence of VRE infection or colonization in hospital- or in community-based patients.

Therefore, the present study was started to determine the prevalence of fecal carriage of VRE in hospitalized patients with an increased risk for VRE infection or colonization, and in community-based patients. We determined the susceptibility of VRE for vancomycin, teicoplanin, avoparcin, a glycopeptide available throughout Europe as additive in animal feed [10], and LY333328, a new glycopeptide antibiotic [11]. In order to determine the genetic basis of the

glycopeptide resistance phenotype, PCR assays aiming at the various resistance genes were performed. Moreover, the VRE were typed using pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR (AP-PCR) and ribotyping to determine genetic relatedness of this group of resistant microorganisms.

Materials and Methods

Prevalence study

Five Dutch university hospitals in Rotterdam, Utrecht, Nijmegen and Amsterdam and 4 regional teaching hospitals in Breda and Tilburg participated in the study. Six hundred twenty-four patients that were hospitalized in the following wards were screened for gastro-intestinal carriage of VRE: medical and surgical ICU; thoracic surgical ICU; neurological/neurosurgical ICU; pediatric ICU (surgical, neonatal or general pediatric); and hemato-oncology wards. The prevalence study was carried out in November 1995 and in February 1996. In addition, 200 outpatients attending general practitioners for diarrhea were screened.

Bacterial strains

Sixteen VRE isolated during the prevalence study were analyzed. Four of these 16 strains were isolated at Rotterdam University Hospital (Hospital A). In addition, 11 clinical VRE strains that were isolated in 1995 in Hospital A before the start of the survey were studied.

Culture and identification

Stool specimens or rectal swabs from all patients were cultured in a selective, aesculin-containing enrichment broth [12,13], supplemented with 50 mg/L cephalexin and 75 mg/L aztreonam (Bristol-Myers Squib, Princeton, NJ). All aesculin-positive broth cultures were subcultured on a new elective agar designed for isolation of *E. faecium* [14], with and without 6 mg/L vancomycin, and on Columbia bloodagar. In a pilot study this procedure proved very convenient and easy since all broth cultures containing enterococci did turn black; all other broth cultures could be disregarded without further processing. All enterococcus-like, arabinose-fermenting as well as arabinose-non-fermenting colonies were sub-cultured. A presumptive identification of *Enterococcus* was

made on the basis of colonial morphology, Gram stain, catalase, PYRase (Difco laboratories, Detroit, Mich.) and Lancefield group D antigen [15]. Definitive identification was done by API 32 rapid system (BioMerieux, Marcy l'Etoile, France). *E. gallinarum* was identified by digestion of DNA with *Sma*I and pulsed-field gel electrophoresis. Strains with all DNA-fragments of <200kb were identified as *E. gallinarum* [16].

Susceptibility testing

Resistance to vancomycin was detected by E-test (AB biodisk, Solna, Sweden) [17]. An inoculum of 0.5 McFarland and Mueller-Hinton agar (Difco) were used. Plates were read after incubation at 37°C for 24h. and the E-test MICs were rounded to the nearest higher doubling dilution. All vancomycin-resistant (MIC>4 mg/L) enterococci were subjected to further susceptibility tests using standard agar dilution and broth dilution methods according to NCCLS guidelines [18]. *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as reference strains. The following glycopeptide agents were tested: vancomycin (Eli Lilly, Indianapolis, Ind.), teicoplanin (MMDRI-Lepetit Research Center, Gerenzano, Italy), avoparcin (Roche, Basel, Switzerland) and LY333328 (Eli Lilly).

DNA isolation

DNA was isolated according to Boom et al [19]. The strains were grown overnight at 37°C on Brucella bloodagar plates. Colonies were suspended in TEG buffer (25 mM Tris-HCl, pH 8.0; 10 mM EDTA and 50 mM glucose). A lysozyme solution (10 mg/L) was added and this mixture was incubated for one hour at 37°C. Guanidine-hydrothiocyanate was added for cell lysis and Celite (Janssen Pharmaceuticals, Beerse, Belgium) was used for DNA binding. DNA was eluted with 10 mM Tris-HCl (pH 8.0). DNA concentration was estimated by electrophoresis on a 1% agarose gel (Hispanagar; Sphaero Q, Leiden, The Netherlands) containing ethidium-bromide in the presence of known quantities of lambda DNA.

PCR assay for VanA, VanB and VanC genes

The PCR assays were performed as described earlier by Dutka-Malen et al. [20]. Approximately 10-100 ng (10 µl) of DNA was added to a PCR mixture (90 µl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 %

gelatine, 0.1% Triton X-100, 0.2 mM of the 4 deoxyribonucleotide triphosphates, 1.2 units of Taq DNA polymerase (Sphaero Q, Leiden, The Netherlands). Four different primer couples (vanA, vanB, vanC1 and vanC2 [20]) were used in the assay (50 pmol of each primer per reaction). Amplification of DNA was performed in a Biomed model 60 thermocycler (Biomed, Theres, Germany), using predenaturation at 94° C for 2 minutes, followed by 30 cycles of 1 minute at 94° C, 1 minute at 54° C and 1 minute at 72° C. Amplicons were analyzed by electrophoresis on a 1% agarose gel (Gibco BRL, Brussel, Belgium) containing ethidium-bromide in the presence of a 100 bp ladder.

Ribotyping

Restriction digestion of 20 µl (5 µg) samples of DNA was done by overnight incubation at 37° C with *Eco*RI (Boehringer GmbH, Mannheim, Germany). DNA fragments were separated by electrophoresis on a 1% agarose gel for 16 hours (30 V, 200 mA). Southern transfers of the gel with *Eco*RI digested DNA were made by capillary blotting to a nylon membrane (Hybond N+, Amersham, UK). The blots were hybridized with a 16S rRNA riboprobe. The probe was synthesized by PCR-mediated amplification of the ribosomal genes of *E. coli*. The amplicon was purified by Qiaquick procedures (Westburg, Leusden, The Netherlands) and labeled ECL-kits (Amersham). Further processing (hybridization, washing, and development) was done according to the ECL guidelines.

AP-PCR

AP-PCR was performed as described before [21]. Approximately 5-50 ng (10 µl) of DNA was added to a PCR mixture (40 µl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatine, 0.1% Triton X-100, 0.2 mM of the deoxyribonucleotide triphosphates, 1.2 units of Taq DNA polymerase. Four different primers were used in separate assays (50 pmol of primer per reaction; ERIC-1R, 5'-ATG TAA GCT CCT GGG GAT TCA C-3'; ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3'; AP-1, 5'-GGT TGG GTG AGA ATT GCA CG-3'; AP-7, 5'-GTG GAT GCG A-3'. Amplification of DNA was performed in a Biomed model 60 thermocycler (Biomed, Theres, Germany), using predenaturation at 94° C for 4 minutes, followed by 40 cycles of 1 minute at 94° C, 1 minute at 25° C and two minutes at 74° C. Banding patterns were visualized after electrophoresis on a 1% agarose gel, containing ethidium-bromide in the presence of a 100 bp ladder.

Banding patterns were interpreted upon visual inspection. Different types were identified on the basis of even a single differentiating DNA fragment. Differences in ethidium bromide staining intensity were ignored.

Pulsed Field Gel Electrophoresis

Ten colonies of an overnight culture, grown on bloodagar, were suspended in 100 µl EET buffer (100 mM Na2EDTA, 10 mM EGTA, 10 mM Tris-HCl [pH 8.0]). This suspension was mixed with 100 µl of 1% agarose (Incert agarose; FMC Co., Bioproducts, Rockland, Maine) and transferred into sample plug molds (final agarose concentration, 0.5%). The plugs were incubated for four hours at 37°C in 1 ml EET buffer containing 10 mg of lysozyme (Sigma Chemicals Co., St. Louis, and Mo). This lysis solution was replaced for a 1 ml EET buffer solution containing 1 mg proteinase K and 1% SDS for a further overnight incubation at 37° C. The plugs were washed six times (30 minutes each at room temperature) in TE solution (10 mM Tris-HCl [pH 8.0], 1mM EDTA). To digest the DNA, a 5 mm slice of the sample plug was placed in a TE solution (10 mM Tris-HCl [pH 8.0], 0.1mM EDTA) with 40 U of SmaI (Boehringer GmbH) and incubated overnight at 25° C. The plugs were loaded on 1% agarose gel (SeaKem GTG agarose; FMC) in 0.5x TBE [22]. Electrophoresis was performed using a CHEF DR II apparatus (Bio-Rad, Richmond, Calif.), programmed in the auto-algorithm mode; block 1: runtime 8 hours, switch time: 0.5-15 seconds and block 2: runtime 10 hours, switch time 15-30 seconds. The gels were stained with ethidium-bromide for 15 minutes and destained in distilled water for 1 hour before photography. All gels were inspected visually by two different investigators. Profiles were designated by a different capital letter any time a distinct (4 or more bands difference) pattern was obtained. Isolates with identical profiles were assigned the same letter. Isolates that differed by 1-3 bands, consistent with a single genetic event, were assigned a subtype [23].

Statistical analysis

Fisher's two-tailed test was used to assess differences between frequencies of isolation of enterococci in the two different patient populations.

Results

Three hundred six (49%) of the 624 hospitalized patients and 161 (80%) of 200 community-based patients carried enterococci in the gastro-intestinal tract ($p<0.01$). Of the 306 enterococci isolated from hospitalized patients, 132 (43%) were identified as *E. faecium.* Out of 161 enterococci from patients outside the hospital, 52 (32%) were identified as *E. faecium* ($p< 0.05$). Thus, *E. faecium* was isolated from 132/624 (21%) of the hospitalized patients and from 52/200 (26%) of the community-based patients ($p> 0.05$). VRE were isolated from 12 (2%) of the 624 hospitalized patients and from 4 (2%) of the 200 community-based patients. Fifteen VRE were identified as *E. faecium*; one was identified as *E. faecalis.* Fifteen (8%) of 184 *E. faecium* strains isolated in the prevalence study were vancomycin-resistant. In addition, 11 strains of VRE were isolated in Hospital A at times separate from the prevalence study period. Nine were identified as *E. faecium*, two as *E. gallinarum*. Thus, 27 VRE were available for further studies.

Table 1: *In-vitro activity of four glycopeptide agents against 27 VRE*[a]

	strain		MIC (mg/L)[b]				*Van* genotype
	number	species	Van	Tei	Avo	LY333328	
clinical isolates[c]	10-a	*E. faecium*	>256	>256	>256	1	A
	10-b	*E. faecium*	>256	>256	>256	0.5	A
	10-c	*E. faecium*	>256	>256	>256	0.25	A
	10-d	*E. faecium*	>256	>256	>256	0.5	A
	10-e	*E. faecium*	>256	128	>256	0.5	A
	10-f	*E. faecium*	>256	>256	>256	0.5	A
	10-g	*E. faecium*	>256	>256	>256	0.25	A
	10-h	*E. faecium*	>256	>256	>256	0.5	A
	10-i	*E. gallinarum*	8	0.5	8	0.25	C1
	10-j	*E. faecium*	8	0.5	8	0.25	B
	10-k	*E. gallinarum*	8	0.5	8	0.25	C1
survey isolates	11-l[c]	*E. faecium*	>256	>256	>256	0.25	A
	12-m[c]	*E. faecium*	>256	>256	>256	0.125	A
	12-n[c]	*E. faecalis*	>256	>256	>256	0.25	A
	12-o[c]	*E. faecium*	>256	>256	>256	0.125	A
	21-p	*E. faecium*	>256	>256	>256	0.125	A
	22-q	*E. faecium*	>256	>256	>256	1	A
	22-r	*E. faecium*	>256	>256	>256	0.25	A
	22-s	*E. faecium*	>256	128	>256	0.25	A
	31-t	*E. faecium*	>256	>256	>256	0.25	A
	32-u	*E. faecium*	>256	>256	>256	0.125	A
	42-v	*E. faecium*	>256	>256	>256	0.125	A
	52-w	*E. faecium*	>256	>256	>256	0.125	A
	62-x	*E. faecium*	>256	>256	>256	0.125	A
	62-y	*E. faecium*	>256	>256	>256	0.25	A
	62-z	*E. faecium*	>256	>256	>256	0.25	A
	62-$_\alpha$	*E. faecium*	>256	>256	>256	0.5	A

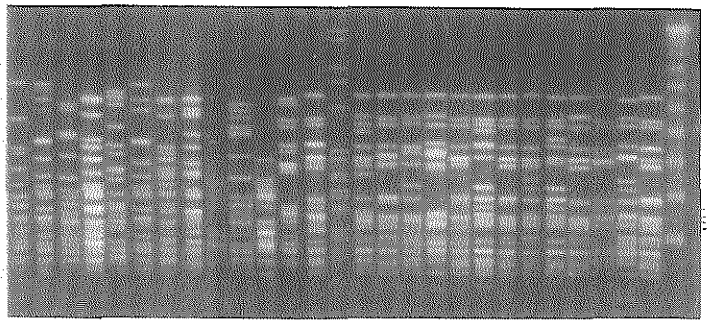
[a] using a standard NCCLS broth dilution method; [b] Van, vancomycin; Tei, teicoplanin; Avo, Avoparcin
[c] strains isolated in Hospital A

Susceptibilities of the 27 VRE for vancomycin, teicoplanin, avoparcin and LY333328 and the resistance genotype are presented in Table 1. Complete cross-resistance was found between vancomycin and avoparcin. LY333328, however, was 250->1000 -fold more active against *VanA* VRE compared to vancomycin. Major discrepancies were observed between the MICs of LY333328 that were found using agar dilution compared to broth dilution: on agar, the MIC_{90} of LY333328 against *VanA* VRE was 4 mg/L (range 0.25-4 mg/L) compared to 0.5 mg/L (range 0.125-1 mg/L) in broth. We did not observe such differences with the other glycopeptide agents. Twenty-four of the 27 VRE, including all VRE from the prevalence study, had the *VanA* genotype; one had the *VanB*, and two had the *VanC1* genotype. All *VanA E. faecium* had MICs of vancomycin > 256 mg/L and teicoplanin > 64 mg/L. The *VanB* and *VanC1* strains had a vancomycin MIC of 8 mg/L and a teicoplanin MIC of 0.5 mg/L.

Table 2: *Overview of PFGE, AP-PCR and Ribotyping of 27 VRE*

	strain number	Ribotype	PFGE	AP-PCR[a]
clinical isolates[b]	10-a	A	A	A
	10-b	B	B	B
	10-c	A	C	C
	10-d	A	D	D
	10-e	A	E	E
	10-f	B	F	B
	10-g	C	G	F
	10-h	A	H	G
	10-i	D	I	H
	10-j	A	J	I
	10-k	D	K	H
survey isolates	11-l[b]	E	L	J
	12-m[b]	A	M	K
	12-n[b]	F	N	L
	12-o[b]	C	O	M
	21-p	A	M^1	M
	22-q	C	P	K
	22-r	A	Q	K
	22-s	A	M^1	M
	31-t	A	R	N
	32-u	A	M^1	M
	42-v	C	S	O
	52-w	A	T	N
	62-x	A	U	M
	62-y	G	V	P
	62-z	A	W	Q
	62-α	A	M^1	K

[a] based on AP-1 and ERIC-2 primers
[b] strains isolated in Hospital A



Location	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 3 3 4 5 6 6 6 6
Screening	0 0 0 0 0 0 0 0 0 0 0 1 2 2 2 1 2 2 2 1 2 2 2 2 2 2 2
Strain	A B C D E F G H I J K L M N O P Q R S T U V W X Y Z α

Figure 1: *Restriction endonuclease pattern obtained by PFGE with Sma1 for 27 strains of VRE isolated from hospital- and community-based patients in The Netherlands. From left to right, the strains appear in the lanes in the same order in which they are listed in Tables 1 and 2. Each strain has a two digital/one letter code corresponding to the location (1, Rotterdam; 2, Amsterdam; 3, Breda; 4, Utrecht; 5, Nijmegen; 6, community), the screening (0, routine isolates from hospital A; 1, prevalence study November 1996; 2, prevalence study February 1997), and a strain letter code corresponding to the order of the strains listed in Tables 1 and 2. A 50 Kb ladder (bio-Rad, Veenendaal, The Netherlands is shown in the lane on the right as molecular size standard*

The restriction endonuclease patterns obtained by PFGE with *SmaI* for 27 VRE are shown in Figure 1. An overview of all typing results is given in Table 2. The discriminatory power of AP-PCR with primers AP-7 and ERIC-1 was low compared to primers AP -1 and ERIC-2. Therefore, only the results of AP-PCR with AP-1 and ERIC-2 are presented in Table 2. Analysis of all 27 VRE revealed 23 different patterns by PFGE, 17 by AP-PCR analysis with primers AP-1 and ERIC-2 and only 7 by ribotyping. Some strains that were indistinguishable by AP-PCR were unrelated by PFGE (e.g. strain 21-p and 12-o). Vice versa, AP-PCR was able to distinguish strains that appeared highly related by PFGE (e.g. strain 32-u and 62-α).

PFGE of 15 strains from Hospital A yielded 15 different patterns. PFGE of 16 VRE from the prevalence study (including 4 strains from Hospital A) yielded 12 different patterns. Five strains isolated from patients hospitalized in three

different hospitals appeared closely related (Table 2). These 5 strains could be divided into 2 different subtypes by AP-PCR. Therefore, the combination of PFGE and AP-PCR demonstrated genetic unrelatedness in 13/16 VRE from the survey.

Discussion

The rapid emergence of resistance in enterococci and the increasing incidence of colonization and infection with VRE have become a health care issue that has caused serious concern to physicians and health authorities alike [8]. This study documents the prevalence of intestinal colonization of selected patients from ICUs and hemato-oncology wards as well as of general practice patients in The Netherlands. Enterococci were found in 49% of the inpatients and in 80% of the outpatients. This proportion of hospitalized patients who carry enterococci is lower than found in previous studies, where 75-90% of the patients carried these microorganisms [4, 24]. These latter studies screened unselected hospitalized patients. One can speculate as to whether greater use of penicillins like amoxicillin or amoxicillin/clavulanic acid combination, may have occurred in our selected group of patients and, thus, may have influenced the prevalence of enterococci isolated from the gastrointestinal tract. We isolated *E. faecium* in 21% of the inpatients and 26% of the outpatients, which is in agreement with previous findings of *E. faecium* in 20-40% of stool cultures [4, 25]. VRE were isolated in 2% of the community-based patients. Several European studies have reported similar frequencies in the community [26,27]. However, a much higher frequency has been reported in a Belgian study [28]. In the latter study, 11 (28%) of 40 healthy community-based volunteers who were not health care workers and had not received antibiotics for at least 1 year, were colonized with VRE. The results of North-American studies performed in the Houston metropolitan area, however, contrast with the European data since VRE appear to be absent in healthy persons in this geographic area [29]. The presence of VRE in the community in Europe parallels the colonization of animals with these resistant organisms [6]. Several studies have now reported the absence of VRE in animals and in the community in the United States, in contrast with the high frequencies in hospitals [30-32]. Some authors, however, have cautioned against comparing the results of the above mentioned studies, since differences in

methodology could, at least in part, explain the observed differences in isolation rates [29].

Since ICU patients and patients in oncology wards were found to be at increased risk for VRE infection or colonization [8], we decided to select these patients for our inpatient survey. The isolation rate in these hospitalized patients was 2% and, therefore, similar to the isolation rate in outpatients. This is roughly in agreement with a recent Belgian study where it was shown that 3.5% of hospitalized patients were VRE carrier [32]. In Finland, Suppola *et al* [24] investigated hospitalized patients with hematological malignancies and reported a VRE prevalence of 2%.

We analyzed the genetic relatedness of the 27 VRE strains by PFGE and AP-PCR. In previous studies PFGE has been shown to be the most discriminating typing technique for VRE and this technique is now considered the gold standard [7, 33]. Recently, however, AP-PCR has proven a powerful typing tool as well. Results of PFGE and AP-PCR are often in concordance [34]. In our study, however, PFGE was more discriminatory compared to AP-PCR. Combining the data generated by the two methods, we demonstrated genetic unrelatedness of 13/16 VRE strains isolated during the survey and of all 15 strains that were isolated in hospital A. No evidence exists for major inter- or intra-hospital spread of VRE in The Netherlands. This observation is remarkable since no special infection-control measures to prevent VRE transmission were in vigor in the participating hospitals at the time of the survey. Together with the observed isolation rate of 2% in the community-based patients, it is suggested that VRE in hospitalized patients may have originated from presently unknown sources in the community. The gastro-intestinal tract is probably the major reservoir in men, from which subsequent infection can eventually develop. This is in agreement with a recent report from New York [22]. Food has been proposed as a source [5, 35]. Others have put forward pets and other domestic animals [36, 37]. Furthermore, the use of antibiotics as feed additives for growth enhancement in animals may be associated with the emergence of VRE [27]. An example of such a growth-promoting agent is avoparcin, a drug that has been used in The Netherlands for a long time. The pig, poultry and calf production is an area of important economic activity in The Netherlands. To date, this country is one of the leading exporters of consumer poultry products in the world, after the US and France [38]. Although official figures are not available, it is clear that

avoparcin has been used in this country on a very large scale. Preliminary results of a nationwide VRE prevalence study in poultry suggest that approx. 80% of the consumer poultry at retail level is colonized with VRE, possibly as a result of unrestricted use of avoparcin in the poultry industry [39]. Thus, the use of oral glycopeptide antibiotics in the bio-industry should be strongly discouraged. Recently, the European Community committed itself to a cautious approach and banned all use of avoparcin as feed additive in animals by 1 April 1997 [Directive 97/6/EC of January 30th 1997]. The emergence of VRE has resulted in an increase in the incidence of infections caused by these organisms that can not be treated with currently available antimicrobial agents [40]. LY333328 is a new semisynthetic glycopeptide that has been reported to increase activity against vancomycin-resistant Gram-positive microorganism's [41]. In our study, LY333328 was found to posses greatly enhanced activity against VRE. In general, the MICs were 25 - 1000 -fold lower against VRE compared to vancomycin. These data are in agreement with an earlier report [11]. Surprisingly, the MICs of LY333328 obtained with an agar dilution method were 4-8 fold higher than with a broth dilution method, for which we do not have an explanation. This phenomenon has recently been reported by others [42]. The results, however, indicate that LY333328 is a promising new drug that deserves further evaluation. In conclusion, we have shown in a multicenter study that VRE can be isolated in hospitalized and in community-based patients in The Netherlands, at a frequency of 2%. Second, these strains appear unrelated and, therefore, no evidence exists for major inter- or intra-hospital spread of VRE strains in our country. Third, our data suggest that VRE be acquired outside the hospital environment. Further studies are warranted to elucidate the origin and the epidemiology of vancomycin resistance. In countries with a large animal livestock, including The Netherlands, where large quantities of feed additives are used, it seems wise to strongly discourage the use of oral glycopeptides not only in man, but in the bio-industry as well.

References

1. Spera RV, Farber BF: Multiply-resistant *Enterococcus faecium*: the nosocomial pathogen of the 1990s. JAMA (1992) 268:2563-2564.
2. Emori TG, Gaynes RP: An overview of nosocomial infections, including the role of the microbiology laboratory. Clin. Microbiol. Rev. (1993) 6:428-442.
3. Murray BE: The life and times of the enterococcus. Clin. Microbiol. Rev. (1990) 3:46-65.

4. Noble CJ: Carriage of group D streptococci in the human bowel. J. Clin. Path. (1978) 31:1182-1186.
5. Murray BE: Editorial response: What can we do about vancomycin-resistant enterococci. Clin. Infect. Dis. (1995) 20:1134-1136.
6. Handwerger S, Raucher B, Altarac D, Monka J, Marchione S, Singh KV, Murray BE, Wolff J, Walters B: Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin and gentamicin. Clin. Infect. Dis. (1993) 16:750-755.
7. Montecalvo MA, Horowitz H, Gedriss C, Carbonaro C, Tenover F, Issah A, Cook P, Wormser P. Outbreak of a vancomycin-, ampicillin- and aminoglycoside-resistant *Enterococcus faecium* bacteremia in an adult oncology unit. Antimicrob. Agents Chemother. (1994) 38:1363-1367.
8. HICPAC: Recommendation for preventing the spread of vancomycin resistance: Recommendation of the Hospital Infection Control Practices Advisory Committee (HICPAC). Am. J. Infect. Control (1995) 23:87-94.
9. Edmond MB, RP Wenzel RP, Pasculle W: Vancomycin-resistant *Staphylococcus aureus:* Perspectives on measures needed for control. Ann. Intern. Med. (1996) 124:329-334.
10. Gliddon MJ, Poole CA, Johannsen FH, Bucher E, Thalman A, Dresbach C, Abramowski B: Microbiological assay of avoparcin in animal feed and pre-mixes-cooperative study. Analyst. (1992) 117:1401-1405.
11. Swalbe RS, McIntosh AC, Qaiyumi A, Johnson JA, Johnson RJ, Furness KM, Holloway WJ, Steele-Moore L: In vitro activity of LY33328, an investigational glycopeptide antibiotic, against enterococci and staphylococci. Antimicrob. Agents Chemother. (1996) 40: 2416-2419.
12. Anonymous: Kanamycin aesculin azide (KAA) agar. Int. J. Food Microbiol. (1987) 5: 731-734.
13. Mossel DAA, Bijker PGH, Eelderink I: Streptokokken der Lancefield-Gruppe D in lebensmitteln und trinkwasser- ihre bedeutung, erfassung und bekämpung. Arch Lebensmittelhyg. (1978) 29:121-127.
14. Facklam R, Pigott N, Franklin R, Elliott J: Evaluation of a three disk test for identification of *Enterococci, Leuconostoc* and *Pediococcus*. J. Clin. Microbiol. (1995) 33:885-887.
15. Ford M, Perry JD, Gould FK: Use of Cephalexin-Aztreonam-Arabinose Agar for selective isolation of *Enterococcus faecium*. J. Clin. Microbiol. (1994) 32: 2999-3001.
16. Donabedian S, Chow JW, Shlaes DM, Green M, Zervos MJ: DNA hybridization and contour-clamped homogeneous electric field electrophoresis for identification of enterococci to the species level. J. Clin. Microbiol. (1995) 33:141-145.
17. Schulz JE, Sahm DF: Reliability of the E test for detection of ampicillin, vancomycin, and high-level aminoglycoside resistance to *Enterococcus* spp. J. Clin. Microbiol. (1993) 31:3336-3339.
18. National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. (1993) 3rd ed. Approved standards. M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
19. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van der Noordaa J: Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. (1990) 28:495-503.
20. Dutka-Malen S, Evers S, Courvalin P: Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. (1995) 33:24-27.
21. Van Belkum A, Kluytmans J, Van Leeuwen W, Bax R, Quint W, Peters E, Fluit A, Vandenbroucke-Grauls C, Van den Brule A, Koeleman H, Melchers W, Meis J, Elaichouni A, Vaneechoutte M, Moonens F, Maes N, Struelens M, Tenover F, Verbrugh HA. Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. (1995) 33:1537-1547.
22. Papanicolaou GA, Meyers BR, Meyers J, Mendelson MH, Lou W, Emre S, Sheiner P, Miller C: Nosocomial infections with vancomycin-resistant *Enterococcus faecium* in Liver transplant recipients: risk factors for acquisition and mortality. Clin. Infect. Dis. (1996) 23:760-766.
23. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B: Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. (1995) 33:2233-2239.

24. Suppola JP, Volin L, Valtonen VV, Vaara M: Overgrowth of *Enterococcus faecium* in the feces of patients with hematological malignancies. Clin. Infect. Dis. (1996) 23:694-697.
25. Benno Y, Suzuki K, Narisawa K, Bruce WR, Mitsuoka T: Comparison of the fecal microflora in rural Japanese and urban Canadians. Microbiol. Immunol. (1986) 30:521-532.
26. Jordens JZ, Griffiths DT: Faecal carriage and nosocomial spread of vancomycin-resistant *Enterococcus faecium*. J. Antimicrob. Chemother. (1994) 34:515-528.
27. Klare I, Heier H, Claus H, Reissbrodt R, Witte W: *VanA*-mediated high-level glycopeptide resistance in *Enterococcus faecium* from animal husbandry. FEMS Microbiol. Let. (1995) 125:165-172.
28. VanderAuwera P, Pensart N, Korten V, Murray BE, Leclercq R: Influence of oral glycopeptides on the fecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J. Infect. Dis. (1996) 173:1129-1136.
29. Coque TM, Tomakayo JF, Ricke SC, Okhuysen PC, Murray BE. Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob. Agents Chemother. (1996) 40:2605-2609.
30. Knudtson LM, Hartman PA: Antibiotic resistance among enterococcal isolates from environmental and clinical sources. J. Food Protection (1993) 56:489-492.
31. Thal LA, Chow JW, Mahayni R, Bonilla H, Perri MB, Donabedian SA, Silverman J, Taber S, Zervos MJ: Characterization of antimicrobial resistance in enterococci of animal origin. Antimicrob. Agents Chemother. (1995) 39:2112-2115.
32. Gordts B, van Landuyt H, Ieven M, Vandamme P, H. Goossens H: Vancomycin-resistant enterococci colonizing the intestinal tract of hospitalized patients. J. Clin. Microbiol. (1995) 33:2842-2846.
33. Miranda AG, Singh KV, Murray BE: DNA fingerprinting of *E. faecium* by pulsed-field gel electrophoresis may be a useful epidemiological tool. J. Clin. Microbiol. (1991) 29:2752-2757.
34. Barbier N, Saulnier P, Chahaty E, Dumontier S, Andremont A: Random amplified polymorphic DNA typing versus pulsed-field gel electrophoresis for epidemiological typing of vancomycin-resistant enterococci. J. Clin. Microbiol. (1996) 34:1096-1099.
35. Donnelly JP, Voss A, Witte W, Murray BE: Does the use in animals of antimicrobial agents, including glycopeptide antibiotics, influence the efficacy of antimicrobial therapy in humans? J. Antimicrob. Chemother. (1996) 37:389-390.
36. Devriese LA, Ieven M, Goossens H, Vandamme P, Pot B, Hommez J, Haesebrouck F: Presence of vancomycin-resistant enterococci in farm and pet animals. Antimicrob. Agents Chemother. (1996) 40:2285-2287.
37. Van Belkum A, Van den Braak N, Thomassen R, Verbrugh HA, Endtz HPh: Vancomycin-resistant enterococci in cats and dogs. Lancet (1996) 348:1038-1039.
38. Elward R: Personal communication (Product Boards for Livestock, Meat and Eggs; The Netherlands).
39. Vliegenthart J: Personal communication.
40. Jones RN, Sader HS, Erwin ME, Anderson SC, and the Enterococcus Study Group: Distribution of vancomycin resistant enterococci (VRE) genotypes differ in ill versus colonized patients. (1995) abstr. E37, p.92. *In* Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington D.C.
41. Nicas TL, Mullen DL, Flokowitsh JE, Preston DA, Snyder NL, Stratford RE, Cooper RDG: Activity of the semisynthetic glycopeptide LY191145 against vancomycin-resistant enterococci and other Gram-positive bacteria. Antimicrob. Agents Chemother. (1995) 39:2585-2587.
42. Jones RN, Barrett MS, Erwin ME: In vitro activity and spectrum of LY333328, a novel glycopeptide derivative. Antimicrob. Agents Chemother. (1997) 41:488-493.

CHAPTER 5

Prevalence and Determinants of Fecal Colonization with Vancomycin-Resistant Enterococci in Hospitalized Patients in The Netherlands

Nicole van den Braak[1*], MSc; Alewijn Ott[2], MD; Alex van Belkum[1], PhD, PhD; Jan A.J.W. Kluytmans[3,4], MD; Johannes G.M. Koeleman[3,5], MD; Lodewijk Spanjaard[3,6], MD; Andreas Voss [3,7], MD; Annemarie J.L. Weersink[3,8], MD; Christina M.J.E. Vandenbroucke-Grauls[3,5], MD; Anton G.M. Buiting[9], MD; Henri A. Verbrugh[1,3], MD; Hubert Ph. Endtz[1], MD

Department of Medical Microbiology & Infectious Diseases, Erasmus University Medical Center Rotterdam[1], Department of Epidemiology and Biostatistics, Erasmus University Rotterdam[2], Working Party Hospital Infection Epidemiology Netherlands (WHEN) [3] and Ignatius Hospital[4], Breda, University Hospital Vrije Universiteit[5] and Academic Medical Center[6], Amsterdam, University Hospital, Nijmegen7, University Hospital, Utrecht[8] and Elisabeth Hospital, Tilburg[9], The Netherlands

Infection Control and Hospital Epidemiology (2000) 21:520-524

Abstract

We determined the prevalence and determinants of vancomycin resistant enterococci (VRE) fecal carriage in Intensive-Care Unit (ICU)-, Hematology-Oncology (HO)- and hemodialysis patients in The Netherlands with a descriptive, multi-center study, with yearly one-week point-prevalence assessment between 1995-1998. All patients hospitalized on the testing days in ICUs and HO wards in nine hospitals in The Netherlands were included. Rectal swabs obtained from 1112 patients were screened for enterococci in a selective broth and subcultured on selective media with and without 6 mg/L vancomycin. Resistance genotypes were determined by PCR. Further characterization of VRE strains was done by pulsed field gel electrophoresis (PFGE). We studied possible determinants of VRE colonization with a logistic regression analysis model. Determinants analyzed included sex, age and log-transformed length of prior hospital stay. The results showed that 614/1112 (55%) patients were colonized with vancomycin sensitive enterococci (VSE) and 15/1112 (1.4%) carried VRE. From 1995-1998, no increase in VRE colonization was observed. Eleven strains were identified as *Enterococcus faecium* and four as *Enterococcus faecalis.* All *E. faecium* and one *E. faecalis* carried the *vanA* gene; the other *E. faecalis* strains harbored the *vanB* gene. PFGE revealed that 3 *vanB* VRE isolated from patients hospitalized in one single ICU, were related, suggesting nosocomial transmission. Though higher age seemed associated with VRE colonization, exclusion of patients with the nosocomial strain from the regression analyses decreased this relation to non-significant. Duration of hospital stay was not associated with VRE colonization. VRE colonization in Dutch hospitals is an infrequent phenomenon. Though, nosocomial spread occurs, most observed cases were unrelated, which suggest the possibility of VRE acquisition from outside the hospital. Prolonged hospital stay, age and sex proved unrelated to VRE colonization.

Introduction

Colonization and infection caused by vancomycin-resistant enterococci (VRE) has been reported in hospitalized patients, particularly in the United States, but also in European countries. VRE has emerged as an important cause of nosocomial infections. The prevalence of patients colonized with VRE is still rising in the United States. The Center for Disease Control (CDC) reported an increase of VRE isolated from nosocomial infections in intensive-care units (ICU's) in the United States from 0.4% to 23.2% [1]. The prevalence of VRE in European hospitals over the last ten years remains low [2, 3]. Nosocomial transmission of VRE plays an important role in the United States and hospital outbreaks with clonally related VRE have been described on various occasions [4-6]. In contrast to the observed clonality in the United States, a high degree of heterogeneity is observed among VRE strains isolated in Europe [7, 8] , suggesting that, in the absence of intrahospital spread, VRE are acquired outside the hospital [9-11]. It is important to analyze and to understand the difference in epidemiology of VRE in the USA and Europe [12]. The issue is complex and the cause of the spread of VRE is likely to be multifactorial. However, further accumulation of epidemiological data may be instrumental to the development of guidelines to prevent the further spread of VRE. Therefore, the aim of this study was to survey the prevalence of fecal carriage of VRE in high risk hospitalized patients in The Netherlands and to describe and analyze several demographic data and patient characteristics of VRE colonization.

Materials and Methods

Prevalence study

Five Dutch university hospitals in Rotterdam, Utrecht, Amsterdam (n=2) and Nijmegen and four regional hospitals in Tilburg and Breda participated in this study. The prevalence surveys were carried out in November 1995, February 1996, February 1997 and June 1998. Preliminary results from the surveys in 1995 and 1996 were reported by Endtz et al [10]. Eleven hundred and twelve patients were screened for gastro-intestinal carriage of VRE. All patients hospitalized on the study days in medical, surgical neurosurgical, neurological,

pediatric and neonatal intensive care units (ICU), hematology and oncology (HO), and hemodialysis wards were included.

Isolation an identification

Isolation and identification was done as described by Endtz et al [10]. In brief, stool specimens or rectum swabs from all hospitalized patients on the testing days were screened for enterococci in a selective, esculine enrichment broth. All esculin-positive broth cultures were subcultured on a selective agar, designed for isolation of *E. faecium* [13], with and without 6 mg vancomycin per liter and on a Columbia blood agar plate (Becton and Dickenson, Meylan Cedex, France). A presumptive identification of the *Enterococcus* spp. was made on the basis of colony morphology, Gram stain, catalase and pyrase (Dryslide Pyrkit, Difco Laboratories, Detroit, USA). Definitive identification was done by the RAPID ID32 STREP assay (BioMérieux, 's Hertogenbosch, The Netherlands).

Antimicrobial susceptibility tests

All enterococcal strains were tested for vancomycin susceptibility on Mueller-Hinton agar (DIFCO Laboratories, Detroit, USA) with E-test strips (AB BIODISK, Solna, Sweden) following the instructions of the manufacturer. All plates were incubated at 37°C and read after 24 h.

DNA isolation

Enterococcal DNA was isolated according to Boom et al. [14]. In brief, VRE strains were grown overnight at 37°C on Brucella blood agar plates. Ten colonies of each isolate were mixed and suspended in 75 µl TEG buffer (25 mM Tris-HCl, pH 8.0; 10 mM EDTA and 50 mM glucose). A lysozyme solution (75 µl of 10 mg/ml) was added and this mixture was incubated for one hour at 37°C. Guanidine-hydrothiocyanate was added for cell lysis and Celite (Janssen Pharmaceuticals, Beerse, Belgium) was used for DNA binding. DNA was washed and finally eluted from Celite with 10 mM Tris-HCl (pH 8.0) by incubation at 56°C for 10 minutes. The DNA concentration was estimated by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) containing ethidium-bromide in the presence of known quantities of lambda DNA as references.

VanA, vanB, vanC1 and vanC2 PCR

Diagnostic PCR assays targeting the various resistance genes were performed as described by Dutka Malen et al. [15]. Four different primer couples (*vanA; vanB; vanC1* and *vanC2*) were used in combination with 50 ng enterococcal DNA. Amplification of DNA was performed in a Biomed thermocycler (Model 60, Theres, Germany), using predenaturation at 94° C for 2 minutes, followed by 30 cycles of 1 minute at 94° C, 1 minute at 54° C and 1 minute at 72° C. Amplicons were analyzed by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q, Leiden, the Netherlands) containing ethidium-bromide in the presence of a 100 basepair DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands).

Pulsed field gel electrophoresis

PFGE was performed as described previously [16]. In brief, colonies of an overnight culture, grown on a blood agar plate, were mixed and suspended in EET buffer (100 mM Na_2EDTA , 10 mM EGTA, 10 mM Tris-HCl; pH 8.0). This suspension was mixed with 1% agarose (Incert Agarose: FMC, Rockland, USA). Cells in the plugs were lysed, washed, stabilized and restricted with *SmaI*. Electrophoresis was performed and the gel was stained with ethidium-bromide before photography under UV irradiation. The gels were inspected visually by two different investigators. The PFGE patterns were interpreted according to Tenover *et al* [17]. Since the interpretative guidelines brought forward by Tenover *et al* are mainly for outbreak investigations, the following additional comparison was performed. Data obtained for all VRE were studied in more detail using Gelcompar software (Applied Maths, Gent, Belgium). The PFGE patterns were scanned and Dice analysis of peak positions was executed. UPGMA was applied and the bandwidth tolerance was set critically at 1.2%.

Statistical analysis

On the wards with sporadic cases we studied possible determinants of VRE carriage with multiple regression analysis, including an analysis in which hospital stay was log-transformed, to normalize distribution. Demographic data for the analysis were obtained from anonymous patient records.

Results

Six hundred and fourteen (55%) of the 1112 hospitalized patients carried vancomycin-sensitive enterococci (VSE) in their gastro-intestinal tract. VRE was found in 15/1112 patients (1.4%). The overall prevalence ranged from 0.8% (2/230) in 1998 to 2.7% (7/256) in 1996. Table 1 gives a summary of all VRE isolated during the study. In 1998, for example, VRE were found only in one hospital which represents a local prevalence of 4.4% (2/45). Eleven strains were identified as *E. faecium*, the remaining four strains were *E. faecalis.*

Table 1: *Vancomycin-resistant enterococci (vancomycin MIC > 256 mg/L) isolated during yearly point-prevalence surveys, The Netherlands, 1995 to 1998*

Location	Screening Year	VRE/Patients (%)	Species	Strain code	Teico MIC (mg/L)	*van* genotype	PFGE
Rotterdam	1995	1/88 (1.1%)	*E. faecium*	VRE 1	>256	A	A
	1996	2/72 (2.8%)	*E. faecium*	VRE 2	>256	A	B
			E. faecalis	VRE 3	>256	A	C
Amsterdam (I)	1997	3/55 (5.4%)	*E. faecalis*	VRE 4	1	B	D
			E. faecalis	VRE 5	1	B	D
			E. faecalis	VRE 6	1	B	D
	1998	2/ 45 (4.4%)	*E. faecium*	VRE 7	>256	A	E
			E. faecium	VRE 8	>256	A	E*
Amsterdam (II)	1995	1/68 (1.5%)	*E. faecium*	VRE 9	>256	A	B+
	1996	2/66 (3.0%)	*E. faecium*	VRE 10	>256	A	F
			E. faecium	VRE 11	128	A	B+
Breda	1995	1/36 (2.7%)	*E. faecium*	VRE 12	>256	A	G
	1996	1/32 (3.1%)	*E. faecium*	VRE 13	>256	A	B+
Nijmegen	1996	1/47 (2.1%)	*E. faecium*	VRE 14	>256	A	H
Utrecht	1996	1/46 (2.1%)	*E. faecium*	VRE 1	>256	A	I

Abbreviation : MIC, minimum inhibitory concentration; PFGE pulsed field gel electrophoresis; VRE, Vancomycin Resistant Enterococci

* differs 3 bands from PFGE type E,+ differs 1 band from PFGE type B

All VRE were highly resistant to vancomycin (> 256 mg/L). Of the 15 VRE, 11 *E. faecium* and 1 *E. faecalis* were resistant to teicoplanin (VanA phenotype) and three *E. faecalis* were susceptible (VanB phenotype).

VRE isolates found in this study were analyzed by PFGE (figure 1). Most of the PFGE banding patterns comprised 15 to 20 DNA fragments. Analysis of banding patterns showed that three out of four *E. faecalis* (Figure 1; VRE 4, 5, 6) were genetically identical and two clusters of E. *faecium* (cluster I; VRE 2, 9, 11, 13 and cluster II; VRE 7, 8) were genetically related. The 3 identical *E. faecalis* strains were isolated in an intensive care unit in one hospital in 1997 and thus represent an epidemic strain. The *E. faecium* strains in cluster II were also isolated in 1997 in this hospital. Therefore, it would seem that these isolates might also have resulted from nosocomial transmission. In contrast, the genetically related *E. faecium* strains in cluster I were isolated in 1995 (n=1) and 1996(n=3) in 3 different hospitals.

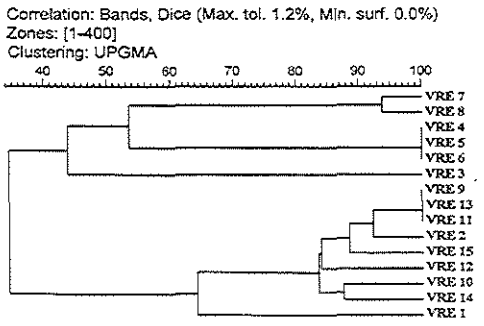


Figure : *Dendogram of vancomycin resitant enterococci isolated from hospitalized patients in one week point-prevalence studies between 1995-1998 in The Netherlands. The origin of VRE 1-15 are listed in Table 1*

Abbreviation: UPGMA, unweighted pair group method using arimethic averages

We identified factors including sex, age and length of hospital stay associated with sporadic VRE colonization (Table2) and studied these determinants with multiple logistic regression analysis. We also ran an analysis in which hospital stay was log-transformed, to normalize distribution. Patients, who were admitted in the ward during the epidemic, were excluded from all regression analyses. Neither duration of hospitalization nor sex correlated with VRE carriage, whether or not the 137 patients under 5 years of age (who had long relatively stay but no VRE) were included in the analysis. There appeared to be a trend of more VRE with higher age (p=0.07). This may however have been based entirely on the

relatively large number of under-fives without VRE, because the relationship disappeared when this group was excluded from analysis. We further investigated the "outbreak" of vanB VRE in 1997 at the ICU in one of the University hospitals. At time of sampling 6/6 patients were colonized with enterococci. Three patients were colonized with VRE (mean age = 70 yrs, median 67 yrs) and 3 were VSE carriers (mean age 58 yrs, median 52 yrs). Here again, no significant difference was found in length of hospital stay between *vanB* VRE and VSE carriers (47 days versus 53 days; p >0.5).

Table 2: *Characterizations of hospitalized patients screened for VRE in yearly point-prevalence surveys,The Netherlands, 1995 to 1998*

	VRE	VSE
Patients, Male	9	353
Female	6	246
Total	15	599
Age (years), Mean	61	43
Range	[29..83]	[0..92]
Median	60	51
Lenght of stay (days), Mean	24	27
Range	[7..40]	[0..219]
Median	15	18

Abbreviation : VRE, vancomycin resisistant enterococci; VSE, vancomycin sensitive enterococci

Discussion

This study was undertaken to determine the prevalence, and the genetic background of VRE in Dutch hospitalized patients and to find determinants associated with VRE carriership in a selected category of hospitalized patients (ICU and hematology wards) in The Netherlands. Fifty-five percent of these patients had enterococcal colonization in the gastro-intestinal tract. Trabusli et al. [18] reported a similar prevalence of enterococci in a group of high-risk

pediatric patients. In contrast, in several studies unselected hospitalized patients were screened for VRE and higher frequencies (75-90%) of patients colonized with enterococci were reported [19, 20].

We isolated VRE from 1.4% of the high-risk patients included in the study. This is somewhat lower than found in other VRE studies in European hospitals. In Belgium 3.5% of the patients in a university hospital carried VRE [7]. In a study in France 4.9% of hospitalized patients were colonized with VRE [8]. However, more recent studies in other European countries report comparable percentages of VRE carriage. In a surveillance study in Germany [2] the VRE carriage rate was 1.5%. In contrast, in seven Norwegian hospitals [3] no *vanA* VRE were found in hospital patients. The authors hypothesized that this was related to the limited use of glycopeptide antibiotics in Norway. The prevalence of VRE in hospitals in The Netherlands as well as in the rest of Europe appears to be rather low and stable over the last ten years. However, prevalence of VRE in the community and in the environment is high. In a previous nation-wide study we showed a high prevalence of 80% VRE in Dutch consumer poultry products [16] and hypothesized that the use of feed additives such as avoparcine may have contributed to the high prevalence of VRE in meat. The European Union banned all use of avoparcine as feed additive in April 1997 and in December 1998; avilamycin, bacitracin, tylosin and virginiamycin were banned [20]. However, controversy still exists over the impact of these measures on the VRE prevalence in humans [21, 22].

PFGE analysis showed that all *vanB E. faecalis* strains were identical; these strains were isolated in one ICU. After the end of the study period two additional patients from the same ICU were reported to be colonized with this VRE clone. This is the first nosocomial clonal outbreak of colonization caused by VRE in the Netherlands. In contrast, when analyzing *E. faecium* we found 4/11 genetically related strain but no geographical relationship. Outbreaks of VRE in Europe occur infrequently. VRE isolated from hospitalized patients as well as from other sources are mostly heterogeneous strains. Interestingly, Willems et al [23] suggested that horizontal transmission of Tn1546 transposon might be an alternative determinant factor driving the spread of vancomycin resistance. Further studies should also take Tn1546 diversity into account; analyses of Tn1546 were not part of the present study.

Several factors can be involved to explain the dissemination of VRE. Goossens [12] suggested that antimicrobial pressure is one of the most important factors for the spread of VRE in the United States. Several risk factors for VRE colonization and infection have been described. Rao et al [24] identified preceding therapy with vancomycin or cephalosporins and prolonged hospital stay as important risk factors. Other studies confirmed that prolonged hospital stay is significantly related to VRE colonization or infection [25-28]. In contrast, we reported that VRE carriage is not associated with prolonged hospital stay in The Netherlands. One may, therefore, assume that, while vancomycin use is possibly the driving force leading to the observed high prevalence in US hospitals, glycopeptide use in animal husbandry in Europe is more likely to be responsible for the occurrence of VRE in Europe. In the absence of any data to refute this hypothesis, it appears that a different approach to intervention is needed. However, it is important to stress that the number of riskfactors analysis in our study was limited, and did not include, for example, analysis of factors such as previous antimicrobial therapy or proximity to known patients with VRE. Therefore, more studies are needed to further clarify the epidemiology of VRE and detect the design of future interventions.

References

1. Martone WJ: Spread of vancomycin-resistant enterococci: Why did it happen in the Unites States? Infect. Control Hosp. Epidemiol. (1998) 19:539-545.
2. Reinert RR, Conrads G, Schlaeger JJ, Werner G, Witte W, Lutticken R, et al: Survey of antibiotic resistance among enterococci in North Rhine-Westphalia, Germany. J. Clin. Microbiol. (1999) 37:1638-1641.
3. Simonsen GS, Andersen BM, Digranes A, Harthug S, Jacobsen T, Lingaas E, et al: Low fecal carrier rate of vancomycin-resistant enterococci in Norwegian Hospital patients. Scan J Infect Dis. (1998) 30:465-468.
4. Centers for Disease Control and Prevention: Nosocomial enterococci resistant to vancomycin in the United States, 1989-1993. *MMWR* (1993) 42:597-599.
5. Chow JW, Kuritza A, Schlaes DA, Green M, Sahm DF, Zervos MJ: Clonal spread of vancomycin-resistant *Enterococcus faecium* between patients in three hospitals in two states. J. Clin. Microbiol. (1993) 31:1609-1611.
6. Pegues DA, Pegues CF, Hibberd PL, Ford Ds, Hooper CP: Emergence and dissemination of a highly vancomycin-resistant *vanA* strain of *Enterococcus faecium* at a large teaching hospital. J. Clin. Microbiol. (1997) 35:1565-1570.
7. Gordts B, Van Landuyt H, Ieven M, Vandamme P, Goossens H: Vancomycin-resistant enterococci colonizing the intestinal tract of hospitalized patients. J. Clin. Microbiol. (1995) 33:2842-2846.
8. Boisivon A, Thibault M, Leclerq R: Colonization by vancomycin resistant enterococci of the intestinal tract of patients in intensive care units from French general hospitals. Clin. Microbiol. Inf. (1997) 3:175-179.
9. Bates J, Jordens Z, Griffiths DT: Farm animals as a putative reservoir for vancomycin resistant enterococcal infection in man. J. Antimicrob. Chemother. (1994) 34:507-516.

10. Endtz HP, Van den Braak N, Van Belkum A, Kluytmans AJW, Koeleman JGM, Spanjaard L, et al: Fecal carriage of vancomycin-resistant enterococci in hospital- and community- based patients in the Netherlands. J. Clin. Microbiol. (1997) 35:3026-3031.
11. Van Belkum A, Van den Braak N, Thomassen R, Endtz HPh: Vancomycin-resistant enterococci in cats and dogs. *Lancet* (1996) 348:1038-1039.
12. Goossens H: Spread of vancomycin-resistant enterococci: differences between the United States and Europe. Infect. Control. Hosp. Epidemiol. (1998) 19:546-550.
13. Anonymous: Kanamycin aesculin azide (KAA) agar. Int. J. Food Microbiol. (1987) 5:731-734.
14. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van der Noordaa J: Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. (1990) 28:495-503.
15. Dutka-Malen S, Evers S, Courvalin P: Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. (1995) 33:24-27.
16. Van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HPh: Molecular Characterization of Vancomycin-Resistant Enterococci from Hospitalized Patients and Poultry Products in The Netherlands. J. Clin. Microbiol. (1998) 36:1927-1936.
17. Tenover FC, Arbeit RD, Goering RV Mickelsen PA, Murray BE, Persing DH, et al: Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. (1995) 33:2233-2239.
18. Trabalusi A, Glover AM, Reising SF, Christie CD: Absence of rectal colonization with vancomycin-resistant enterococci among high-risk pediatric patients. Infect. Control Hosp. Epidemiol. (1998) 2:109-112.
19. Noble CJ: Carriage of group D enterococci in the human bowel. J. Clin. Path. (1978) 31:1182-1186.
20. Swalbe RS, McIntosh AC, Qaiyumi A, Johnson JA, Johnson RJ, Furness KM, et al: In vitro activity of LY33328, an investigational glycopeptide antibiotic, against enterococci and staphylococci. Antimicrob. Agents Chemother. (1996) 40:2416-2419.
21. European Commission: A ban of the use of some antibiotics in animal feed. (Press release 10 December 1998) Brussels, Belgium.
22. Klare I, Badstubner D, Konatbel C, Bohme G, Claus H, Witte W: Decreased incidence of Van-A type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb. Drug Resist. (1999) 5:45-53.
23. Willems RJL, Top J, van den Braak N, van Belkum A, Mevius DJ, Hendriks G, et al: Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. Antimicrob. Agents Chemother. (1999) 43:483-491.
24. Rao GG, Ojo F, Kolokithas D: Vancomycin-resistant gram-positive cocci: risk factors for fecal carriage. J. Hosp. Infect. (1997) 35:63-69.
25. Spera RV, Farber BF: Multidrug-resistant *Enterococcus faecium*. An untreatable nosocomial pathogen. Drugs (1994) 48:678-688.
26. Roghmann MC, Fink JC, Polish L, Maker T, Brewrink J, Morris JG, et al: Colonization with vancomycin-resistant enterococci in chronic hemodialysis patients. Am. J. Kidney. Dis. (1998) 32:254-257.
27. Bhorade SM, Christenson J, Pohlman AS, Arnow PM, Hall JB: The incidence of and clinical variables associated with vancomycin resistant enterococcal colonization in mechanically ventilated patients. Chest (1999) 115:1085-1099.
28. Gin AS, Zhanel GG: Vancomycin–resistant enterococci. Ann. Pharmacother. (1996) 30:615-624.

CHAPTER 6a

Dietary Habits and Gastro-intestinal Colonization by Antibiotic Resistant Microorganisms

Nicole van den Braak, Alex van Belkum, Deborah Kreft, Henri Verbrugh and Hubert Endtz

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands

Adapted from: *Journal of Antimicrobial Chemotherapy (2001) 47:498-500*
Lancet (1997) 350:146-7

The human gastro-intestinal flora is affected by probiotics such as lactobacilli, the use of antibiotics and, last but not least, dietary habits [1]. It appears obvious that intestinal metabolism and mucosal immunity change with type of food intake, which is also correlated with changes in the ratio in which different microorganisms occur [2] . Antibiotics, like food, greatly effect the composition of the gastro-intestinal flora and may select for resistant strains. This does not only happen for patients, but it may also occur when antibiotics are used as growth promoters in modern food-animal production [3]. Meat products have been shown to be colonized with resistant microorganisms [4]. This raises the question whether meat serves as a vector during zoonotic transmission. The question whether antibiotic resistant micro-organisms present in food may persistently colonize the human gastro-intestinal tract can be answered by designing studies that compare the faecal flora of vegetarians and that of non-vegetarians, meat eating controls.

We performed a large study on the presence of vancomycin-resistant microorganisms in the faecal flora of vegetarians in 1997. We obtained rectal swabs from 318 vegetarians (mean age 55±18 years, mean number of meat free years 30±2) and 276 non-vegetarian control individuals (mean age 53±17 years). The swabs were inoculated in Enterococcosel medium (BBL, Cockeysville, USA) in order to select for vancomycin-resistant enterococci (VRE). The results documented a statistically significant difference in the occurrence of low-level VRE such as *Enterococcus casseliflavus* and *Enterococcus gallinarum* in vegetarians versus controls [5]. Briefly, 31 vegetarians carried VRE, a number of 20 *E. casseliflavus* (*vanC2*) and 10 *E. gallinarum* (*vanC1*) was found. Two of these 31 persons were colonized with *E. gallinarum* (*vanC1*) as well as *E. casseliflavus* (*VanC2*). Among 'meat-eating' persons we found that 13 persons were colonized with VRE, 1 with *E. faecium* (*VanA*), 7 with *E. gallinarum* (*vanC1*) and 5 with *E. casseliflavus* (*vanC2*). One person carried two VRE (*E. gallinarum* and *E. casseliflavus*). In conclusion, we found no high level vancomycin resistant VRE in the vegetarian group. In contrast, one VRE (*VanA*) was isolated in the non-vegetarian group (0.4%). Interesting is the difference in colonization by low-level VRE, especially *E. casseliflavus*, in vegetarian versus non-vegetarian persons. Overall, 31/318 (9,7%) and 12/276 (4.3%) ($p<0.05$) VRE were harboring the *vanC* gene. We suggest that vegetarian people have a significantly higher carriage rate of VRE possessing the *VanC* gene. Fortunately, this kind of VRE is nowadays

not associated with clinical infections in human. No association was found between meat consumption and carriage of high level VRE, as opposed to previous analyses by another Dutch group [6]. However, both studies indicated an apparent difference in the colonization rate of antibiotic-resistant microorganisms as a consequence of dietary habits. This emphasizes the need for further investigations. It was suggested that the high rate of low-level VRE carriage in vegetarians could be due to the fact that especially *E. casseliflavus* was found to be associated with plants.

In the same group of vegetarians and controls, lactose-positive Gram-negative (LPGN) rods were cultured from the faecal specimens on MacConkey agar. *Escherichia coli* strains (n=117), identified with the Vitek system, were randomly selected from the LPGN rods isolated from faecal samples of 318 vegetarians. As a control group, 101 additional *E. coli* strains were cultured from the rectal swabs obtained from the 276 non-vegetarians. Susceptibility for various antimicrobial agents was assessed (see table) using the disk diffusion method according to the NCCLS [7]. The antimicrobial agents investigated comprised drugs registrated for use in humans as well as agents used as growth promoter in animal husbandry. In absence of accurate NCCLS guidelines for tylosine, zone diameters were defined as following manufacturers criteria: ≥26 mm, susceptible; 23-25 mm, intermediate; ≤ 22 mm, resistant. The enterococci were also screened for high-level gentamicin and streptomycin resistance (MIC >500 mg/L) with E-tests (Oxoid, Hampshire, England).

The table displays the results of the susceptibility tests performed for all low-level VRE. When the resistance ratios in vegetarians were compared with controls (Fisher's exact tests) there appeared to be significantly more bacitracin intermediate strains in the control group only ($p = 0.0067$). The table also provides an inventory of the antimicrobial susceptibility of the *E. coli* strains. None of the comparisons appear to be significant. However, we do observe a trend towards decreased susceptibility to nitrofurantoin in the control group ($p = 0.06$). Overall, no clear differences seem to exist when the resistance to various antibiotics is assessed in *E. coli* or enterococci from the two groups. However, when the prevalence of resistant *E. coli* in community-based vegetarians and volunteers are compared to resistance figures in *E. coli* from hospitalized patients (Table 1, column on the right) the nosocomial strains are

Table 1 *Antimicrobial resistance as defined by disk diffusion testing of VanC enterococci and E. coli strains isolated from vegetarians and controls.*

	VanC enterococci						*Escherichia coli*								
Antibiotic	***Control strains***			***Vegetarian isolates***			***Control strains***			***Vegetarian isolates***			***Hospital strains***		
	(n=13)			(n=30)			(n=101)			(n=117)			(n=2447)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Norfloxacin	***15***	***46***	***38***	***3***	***43***	***53***	***0***	***0***	***100***	***0***	***0***	***100***	***5***	***0***	***95***
Nitrofurantoin	15	23	62	3	17	80	**0**	**20**	**80**	**0**	**10**	**90**	6	0	94
Trimethoprim	15	0	85	27	10	63	6	0	94	10	0	90	-	-	-
Cefuroxim	100	0	0	100	0	0	0	0	100	0	0	100	4	14	82
Cotrimoxazol	8	0	92	3	0	97	5	1	94	8	0	92	28	0	72
Piperacillin	0	0	100	7	7	87	17	1	83	16	1	83	35	5	60
Amoxicillin	0	0	100	3	0	97	19	0	81	24	2	74	43	1	57
Tylosin	8	0	92	3	0	97	100	0	0	100	0	0	-	-	-
Bacitracin	**0**	**69**	**31**	**0**	**23**	**77**	100	0	0	100	0	0	-	-	-
Gentamicin*	0	0	100	0	0	100	2	0	98	1	0	99	4	0	96
Streptomycin*	8	0	92	3	0	97	29	28	44	22	37	41	-	-	-
Tetracyclin	31	0	69	20	0	80	24	0	76	21	0	79	-	-	-
Spiramycin	15	0	85	17	0	83	100	0	0	100	0	0	-	-	-

The table states percentages of strains resistant (R), intermediately susceptible (I) or fully susceptible (S) towards the antibiotics used. The control collection consisted of 5, 7 and 1 strains of *E. casseliflavus*, *E. gallinarum* and *E. faecium*, respectively. The isolates from the vegetarians were 20 *E. casseliflavus* and 10 *E. gallinarum*. *Values as determined by E tests for the enterococci. The *E. coli* hospital strains were collected in 1999, included are single isolates per patients selected for the most resistant isolate available in the files (-: data not available). Datasets that differ (nearly) significantly are highlighted by bold lettering.

markedly more resistant to nearly all of the antibiotics. Apparently, the impact of hospitalization on the prevalence of antibiotic resistant bacteria is more important than dietary habits.

Our data suggest that the gastro-intestinal flora and the prevalence of drug-resistant bacteria vary with dietary habits. This is particularly clear from the enterococcal colonization of the vegetarian gut: with *vanC* enterococci [5]. Interestingly, *vanC* enterococci from vegetarians are also significantly less susceptible to the antibiotic bacitracin, which has been used in food production animals. We are, however, not aware, of the use of this drug in agriculture. Apart from a trend towards a decreased susceptibility to nitrofurantoin in *E. coli* from vegetarians, strains did not differ in susceptibility from controls. Our data are not in agreement with unexplained and contradictory figures published in The Netherlands three decades ago [8]. The latter study showed higher prevalences of antibiotic resistant *E. coli* strains in vegetarians and babies than in mixed-diet adults. To conclude, although the scope of our study was limited, it is reassuring to note that we failed to detect significant associations between the consumption of meat and antibiotic resistance determinants in the gastro-intestinal flora.

References

1. Gorbach SL: Function of the normal human microflora. Scand. J. Infect. Dis. (1986) 49:17-30.
2. Noack-Loebel C, Kuster E, Rusch V, Zimmermann K: Influence of the different dietary regimens upon the composition of the human fecal flora. Prog. Food Nutr. Sci. (1983) 7:127-131.
3. Endtz HPh, van den Braak N, Verbrugh HA, van Belkum A: Vancomycin resistance: status quo and quo vadis. Eur. J. Clin. Microbiol. Infect. Dis. (1999) 18:683-690.
4. Van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HPh: Molecular characterization of vancomycin resistant enterococci from hospitalized patients and poultry products in The Netherlands. J. Clin. Microbiol. (1998) 36:1927-1932.
5. Van den Braak N, Kreft D, van Belkum A, Verbrugh, Endtz HPh: Vancomycin-resistant enterococci in vegetarians. Lancet (1997) 350:146-147.
6. Schouten MA, Voss A, Hoogkamp-Korstanje JAA: VRE and meat. Lancet (1997) 349:1258.
7. National Committee for Clinical Laboratory Standards: Methods for antimicrobial susceptibility tests for bacteria that grow aerobically. (1997) 4th Edition. Approved Standards, M7A4, Villanova, Pa.
8. Guinee P, Ugueto N, van Leeuwen N: *Escherichia coli* with resistance factors in vegetarians, babies and nonvegetarians. Appl. Microbiol. (1970) 20:531-535.

CHAPTER 6b

Vancomycin Resistant Enterococci in Cats and Dogs

Alex van Belkum, Nicole van den Braak, Ronald Thomassen, Henri Verbrugh and Hubert Endtz

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands

Lancet (1996) 348:1038-1039

In The Netherlands approximately $1,4x10^6$ dogs and $2,0x10^6$ cats are being kept as pets. Altogether these animals produce a quantity of at least 100-200 tons of faeces daily. Most of this is being shed in an environment which is crowded with people and as such might pose a microbiological threat as was recently demonstrated for bacteria occurring in cats and belonging to the species *Salmonella* [1]. The fact that cats use indoor litter trays for defecation can be considered an additional risk factor. In the present era of zoonotic concern, with the recent bovine spongiform encephalitis (BSE) epidemic as the notorious highlight [2], an increased concern is voiced with the hardly controllable administration of antibiotics as food additives in bio-industry [3]. The use of growth promoting antibiotics results in an ecological pressure of multiresistant microorganisms that may reach the large numbers of potential animal reservoirs being kept in the household environment. We, therefore, wished to study the prevalence of vancomycin-resistant enterococci (VRE) in the population of cats and dogs in The Netherlands.

During the last week of July (1996) 24 cats and 23 dogs attending an urban general veterinary practice (Rotterdam, The Netherlands) were sampled in the rectum. Rectal swabs were inoculated into a selective culture medium (Enterococcosel, containing vancomycin and aztreonam), which turns black in the presence of VRE.

Surprisingly, 11/23 (48%) of the dogs and 4/24 (16%) of the cats were colonized with VRE. Six of the dogs (26%) were colonized with *Enterococcus faecium*, which were highly resistant to vancomycin and teicoplanin: all strains harbored the *van*A gene as was confirmed by PCR. The other five dogs were colonized with *Enterococcus gallinarum* harboring the *vanC1* gene, which confers low level resistance to glycopeptides. For two cats, respectively, *vanA* and *vanC1* genotypes were detected by PCR. Taking cats and dogs together, 17% harbored VRE of the *Van*A genotype and 15% carried VRE containing the *Van*C1 gene. Pulsed field gel electrophoresis (PFGE) yielded two pairs of strains containing the *vanA* gene that were genetically identical. Interestingly, in both pairs one was derived from a dog, whereas the other was cultured from a cat. This indicates a lack of host specificity among strains of VRE and suggests cross-colonization from one pet species to another.

This incidence of VRE in pets which exceeds that encountered among the people living in the same geographic locale (2-3%). Although our data are representative of a single region only, it is obvious that domestic pets may be a significant

reservoir for VRE, the relevance of which has to be determined by follow-up analyses including studies looking into the source of these VRE. Colonization of pets may be a consequence of eating raw meat contaminated with VRE. In clinical settings the spread of VRE should be limited as much as possible [4]. By using simple questionnaires it can easily be established whether patients possessing a cat or dog are at an increased likelihood of being colonized by VRE. One of the VRE genotypes shared among dogs and cats was recently found in a human carrier as well (unpublished data). This raises the question which dog poses a greater risk to the average postman: the one that barks or the one that wags its tail?

References

1. Wall PG, Threlfall EJ, Ward LR, Rowe B. Multiresistant *Salmonella typhimurium* DT104 in cats: a public health risk. Lancet (1996) 348:471.
2. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG: A new variant of Creutzfeldt-Jakob disease in the UK. Lancet (1996) 347:921-25.
3. Van den Boogaard AE, Stobberingh EE. Time to ban all antibiotics as animal growth-promoting agents? Lancet (1996) 348:619.
4. The Hospital Infection Control Practices Advisory Committee HICPAC: Recommendations for preventing the spread of vancomycin resistance: Recommendations of the Hospital Infection Control Practices Advisory Committee. Am J Infect Control (1995) 23:87-94.

CHAPTER 7

Molecular Characterization of Vancomycin-Resistant Enterococci from Hospitalized Patients and Poultry Products in The Netherlands

Nicole van den Braak, Alex van Belkum, Marrit van Keulen, John Vliegenthart[1], Henri A. Verbrugh and Hubert Ph. Endtz

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands
Inspectorate for Health Protection, Food Inspection Service Goes, The Netherlands[1]

Journal of Clinical Microbiology (1998) 36:1972-1932

Abstract

Vancomycin-resistant enterococci (VRE) pose an emerging health risk but little is known on the precise epidemiology of the genes coding for vancomycin resistance. To determine whether the bacterial flora of consumer poultry serves as gene reservoir, the level of contamination of poultry products with VRE was determined. VRE were genotyped with pulsed-field gel electrophoresis (PFGE) and transposon structure mapping was done by PCR. The *vanX-vanY* intergenic regions of several strains were further analyzed by sequencing. 242/305 (79%) poultry products were found to be contaminated by VRE. Of these, 142 (59%) were high level vancomycin-resistant *Enterococcus faecium* (VREF). PFGE revealed extensive VREF heterogeneity. Two genotypes were found nationwide on multiple occasions (type A, 22/142 [15%]; type B, 14/142 [10%]). No PFGE-deduced genetic overlap was found, when VREF from humans were compared with the VREF poultry strains. Two *vanA* transposon types were identified among poultry strains. In 59/142 (42%) of the poultry VREF, the size of the intergenic region between *vanX* and *vanY* was ± 1300 bp. This transposon type was not found in human VREF. In contrast, all human strains and 83/142 (58%) of the poultry VREF contained an intergenic region with the size of 543 bp. Comparative sequencing this 543 bp intergenic *vanX-vanY* region demonstrated full sequence conservation. Though preliminary, these data suggest that dissemination of the resistance genes encoded on transposable elements may be of greater importance than clonal dissemination of resistant strains. This observation is important for developing strategies to control the spread of glycopeptide resistance.

Introduction

Colonization and infection by vancomycin-resistant enterococci (VRE) have been reported in hospitalized patients and in the community in various European countries including France [1,2], the United Kingdom [3,4], Belgium [5] and The Netherlands [6]. VRE pose a health risk, especially in patients with severe underlying disease or immunosuppression. In the United States, the prevalence of VRE in hospitalized patients is rising and hospital outbreaks with clonally related VRE have been described [7-10]. In contrast, the prevalence of VRE in hospitals in Europe remains low and a high degree of heterogeneity is observed among the VRE strains. Bates *et al* [11] suggested that European VRE might be more widely disseminated than originally supposed. Furthermore, there are cases on record of the isolation of VRE from animals and from environmental sources in many European countries [11-14]. Paradoxically, VRE have not yet been recovered from animal and environmental sources in the United States [15,16]. The spread of vancomycin-resistance is of considerable concern. Noble *et al* [17] reported in vitro conjugative transfer of high-level vancomycin resistance from *Enterococcus faecalis* to *Staphylococcus aureus*. In response, the Hospital Infectious Control Practices Advisory Committee (HICPAC) in collaboration with the Centers for Disease Control and Prevention (CDC) has developed recommendations to prevent the spread of VRE [18]. Others have proposed control measures in case vancomycin-resistant *S. aureus* should eventually arise [19]. Recently, scientists from Japan and the United States have reported *S. aureus* intermediately resistant to vancomycin isolated from patients [20,21], although this resistance has been shown not to be mediated by *vanA*, *vanB* or *vanC* genes [22].

The increasing use of antimicrobial agents in human medicine and as animal growth promoters has been related to the emergence of VRE [9]. In Europe, antimicrobial agents are widely used as feed additives for growth promotion in animal husbandry [23]. Avoparcin is a glycopeptide antibiotic used for this purpose in poultry and it appears to be associated with the emergence of resistance to glycopeptides in general [11,24,25]. Enterococci belong to the natural intestinal flora of poultry. It is, thus, not unlikely that transmission of VRE occurs through human contact with poultry meat contaminated with resistant bacteria. However, such a transmission route of VRE from poultry to humans has not been unequivocally documented so far. We determined the level of contamination of

poultry products with VRE. The VRE isolated from poultry products were compared with a collection of VRE isolated from humans [6] with regard to their overall genome structure and eventual polymorphism in Tn*1546*, the transposon encoding high-level glycopeptide resistance.

Materials and Methods

Poultry products

A total of 305 poultry products (whole chicken, legs of chicken, chicken breasts or other parts) from either butchers, supermarkets, poulterers or market poulterers were collected by Dutch Food Inspection Services in the following cities: Den Haag, Maastricht, Alkmaar, Amsterdam, Nijmegen, Rotterdam, Leeuwarden, Den Bosch, Goes, Zutphen and Groningen. The sampling period was from June until September 1996.

Isolation of VRE

Approximately 250 g of each poultry product was rinsed in 250 ml Buffered Pepton Water (BPW, Oxoid, Hampshire, England). After overnight incubation of the BPW at 37°C, 1 ml was used to inoculate 9 ml Enterococcosel (BBL, Becton and Dickinson Microbiology Systems, Cockeysville, USA) supplemented with 6 mg/l vancomycin and incubated at 37° C for 24-48 hours [25]. All aesculin positive broth cultures were subcultured on a Kanamycin Aesculin Azide agar (Oxoid, Hampshire, England) [26]. A presumptive identification of the *Enterococcus* spp. was made on the basis of colony morphology, Gram stain, catalase and pyrase (Dryslide Pyrkit, Difco Laboratories, Detroit, USA). Definitive identification was done by Accuprobe (GenProbe, San Diego, USA) and RAPID ID32 STREP (bioMérieux, 's Hertogenbosch, The Netherlands). The identification strips were read after 5 and 24 hours of incubation at 37° C. All strains containing the *vanC1* gene were identified as *Enterococcus gallinarum* [27]. Strains were stored at -80°C in media containing 15% glycerol.

Additional enterococcal strains

Nineteen vancomycin-resistant *Enterococcus faecium* (VREF) and one VR *E. faecalis* from hospitalized patients and 4 VREF from non-hospitalized patients [21]

were also included in the study. All strains were highly resistant to both vancomycin and teicoplanin and possessed the *vanA* gene (see below). *E. faecium* BM4147 (*vanA*), *E. faecalis* V583 (*vanB*), *E. faecalis* ATCC 19433, *E. faecalis* ATCC 29212, *E. gallinarum* BM4147 (*vanC1*), *Enterococcus casseliflavus* CCUG 18657 (*vanC2*), *Streptococcus bovis* ATCC 33317 and *S. aureus* ATCC 29213 were used as reference strains.

Antimicrobial susceptibility tests

All enterococcal strains described above were tested for vancomycin and teicoplanin resistance on a Mueller-Hinton agar (DIFCO Laboratories, Detroit, USA) with E-test strips (AB BIODISK, Solna, Sweden) following the instructions of the manufacturer. All plates were incubated at 37° C and read after 24 h.

DNA isolation

DNA was isolated according to Boom et al. [28]. In brief, all VRE strains were grown overnight at 37° C on Brucella bloodagar plates. Ten colonies were mixed and suspended in 75 µl TEG buffer (25 mM Tris-HCl, pH 8.0; 10 mM EDTA and 50 mM glucose). A lysozyme solution (75 µl of 10 mg/ml) was added and this mixture was incubated for one hour at 37° C. Guanidine-hydrothiocyanate was added for cell lysis and Celite (Janssen Pharmaceuticals, Beerse, Belgium) was used for DNA binding. DNA was washed and finally eluted from Celite with 10 mM Tris-HCl (pH 8.0) by incubation at 56° C for 10 minutes. The DNA concentration was estimated by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) containing ethidium-bromide in the presence of known quantities of lambda DNA as references.

VanA, vanB, vanC1 and vanC2 PCR

Diagnostic PCR assays targeting the various resistance genes were performed as described by Dutka-Malen *et al* [29]. Approximately 10-100 ng (10 µl) of DNA was added to a PCR mixture (90 µl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatine, 0.1% Triton X-100, 0.2 mM deoxyribonucleotide, 1.2 units of Taq DNA polymerase (Sphaero Q, Leiden, the Netherlands). Four different primer couples (*vanA; vanB; vanC1* and *vanC2*, see Table 1 for DNA sequences) were used in the assay (50 pmol of each individual primer per reaction). Amplification of DNA was performed in a Biomed thermocycler (Model

60, Theres, Germany), using predenaturation at 94° C for 2 minutes, followed by 30 cycles of 1 minute at 94° C, 1 minute at 54° C and 1 minute at 72° C. Amplicons were analyzed by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) containing ethidium-bromide in the presence of a 100 basepair DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands).

Transposon mapping by PCR

To study heterogeneity of the *vanA* encoding transposon Tn*1546*, potentially length variable regions within the 10.801 bp genetic element were studied by PCR (Table 1 for primer sequences) [30,31]. Trial experiments were performed for *E. faecium* and *E. faecalis* only, and selection of a limited number of strains derived from either humans or poultry was at random. PCR was performed as described above. Whenever differences were detected in amplicon size, all additional *E. faecalis* and *E. faecium* strains harboring the *vanA* gene were investigated.

Pulsed field gel electrophoresis

Ten colonies of an overnight culture, grown on a bloodagar plate, were mixed and suspended in 100 µl EET buffer (100 mM Na_2EDTA , 10 mM EGTA, 10 mM Tris-HCl; pH 8.0). This suspension was mixed with 100 µl of 1% agarose (Incert Agarose: FMC, Rockland, USA) and pipetted into small plug molds. The cells suspended in the agarose plugs were lysed by incubation for four hours at 37° C in 1 ml EET buffer containing 10 mg of lysozyme (Sigma, Instruchemie, Hilversum, the Netherlands). Next, the lysis solution was replaced by a 1 ml EET buffer solution containing 1 mg proteinase K (dissolved in 10 mM NaCl, 10 mM Tris-HCl; pH 8.0, 1% SDS) and were further incubated at 37° C for 16 hours. The plugs were then washed six times (30 minutes each time at room temperature) with $T_{10}E_1$ solution (10 mM Tris-HCl; pH 8.0, 1mM EDTA). Plugs were then stabilized twice for 30 minutes in 120 µl of 1x restriction buffer solution, and approximately 40 U of the restriction enzyme *Sma*I (Boehringer Mannheim, Mannheim, Germany) was added (incubation 16 hr, 25° C). Electrophoresis (1% SeaKem agarose in 0.5x TBE) was performed using a BioRad CHEF mapper, programmed in the auto-algorithm mode (block 1: runtime 8 hours; switchtime: 0.5-15 seconds and block 2: runtime 10 hours; switchtime 15-30 seconds). The gel was stained with

ethidium-bromide for 15 minutes and then destained in distilled water for 1 hour before photography under UV irradiation.

Table 1: *Nucleotide sequences of PCR primers*

Primer pair	Nucleotide sequences	Size of PCR product (bp)	Reference
DIAGNOSTIC PCR			
A1 A2	5'-GGGAAAACGACAATTGC-3' 5'-GTACAATGCGGCCGTTA-3'	732	[29]
B1 B2	5'-ATGGGAAGCCGATAGTC-3' 5'-GATTTCGTTCCTCGACC-3'	635	[29]
C1 C2	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCCGCCATCATAGCT-3'	822	[29]
D1 D2	5'-CTCCTACGATTCTCTTG-3' 5'-CGAGCAAGACCTTTAAG-3'	439	[29]
TRANSPOSON MAPPING / STRUCTURAL GENES			
VanR VanR1	5'-AGCGATAAAATACTTATTGTGGA-3' 5'-CGGATTATCAATGGTGTCGTT-3'	645	[31]
VanS VanS1	5'-AACGACTATTCCAAACTAGAAC-3 ' 5'-GCTGGAAGCTCTACCCTAAA-3'	1094	[31]
VanH VanH1	5'-ATCGGCATTACTGTTTATGGAT-3' 5'-TCCTTTCAAAATCCAAACAGTTT-3'	943	[31]
VanA VanA1	5'-ATGAATAGAATAAAAGTTGCAATAC-3' 5'-CCCCTTTAACGCTAATACGAT-3'	1029	[31]
VanY VanY1	5'-ACTTAGGTTATGACTACGTTAAT-3' 5'-CCTCCTTGAATTAGTATGTGTT-3'	866	[31]
Orf1A Orf1A1	5'-AGGGCGACATATGGTGTAACA-3' 5'-GGGCGACGGTACAACATCTT-3'	844	[31]
Orf1B Orf1B1	5'-TGGTGGCTCCTTTTCCCAGTTC-3' 5'-CGTCCTGCCGACTATGATTATTT-3'	1007	[31]
Orf1C Orf1C1	5'-ACCGTTTTTGCAGTAAGTCTAAAT-3' 5'-AAACGGGATTTAGAAATAGTTAAT-3'	1066	[31]
Orf2D Orf2D1	5'-CCATTTCTGTATTTTCAATTTATTA-3' 5'-CATAGTTATCACCCTTTCACATA-3'	925	[31]
Orf2E Orf2E1	5'-TTGCGGAAAATCGGTTATATTC-3' 5'-AGCCCTAGATACATTAGTAATT-3'	540	[31]
TRANSPOSON MAPPING / INTERGENIC REGIONS			
VanXY1 VanXY2	5'-AATAGCTATTTTGATTTCCCCGTTA-3' 5'-TCCTGAGAAAACAGTGCTTCATTAA-3'	543	[30]
VanSH1 VanSH2	5'-TAGGGTAGAGCTTCCAGCGATTGC-3' 5'-CTCATCCTGCTCACATCCATAAACA-3'	311	[30]
VanYZ1 VanYZ2	5'-GTTTCCCGGATCAACACATACTA-3' 5'-CCCAGTAGCAGTAAATGGAGTCA-3'	336	[30]

NOTES: * Primers D1 and D2 are specific for the *vanC2* gene.
* Orf1 = transposase / Orf2 = resolvase

The gels were inspected visually by two different investigators. The PFGE patterns were interpreted according to Tenover *et al* [32]. Isolates that differed by 1-3 bands, consistent with a single differentiating genetic event, were assigned a numbered subtype. Four or more band-differences between two strains defined a different genotype. Genotypes determined for all VREF isolated from chicken were compared with the PFGE characteristics determined for VREF isolated from humans [6]. Since the interpretative guidelines brought forward by Tenover *et al* [32] are for outbreak investigations mainly, additional comparisons were performed. Data obtained from a randomly selected group of 48 human- and poultry-derived VRE were studied in more detail using Gelcompar software (Applied Maths, Gent, Belgium). The PFGE patterns were scanned and Dice analysis of peak positions was executed. UPGMA was applied and the bandwidth tolerance was set critically at 1.2%.

Cloning and sequencing

For several strains the amplicon derived from the *vanX-vanY* intergenic region was cloned into the plasmid pCR1 (Invitrogen, Leek, the Netherlands) according to the manufacturers instructions. Clones containing a correctly sized insert were sequenced using cycle sequencing technology and an ABI 373 sequencing machine (ABI, Warrington, Great Britain). Raw sequence data were edited using 373 software (ABI, Warrington, Great Britain).

Results

VRE screening and antimicrobial susceptibility testing

Table 2 summarizes all data gathered for the chicken specimens. Apparently, 242/305 (79%) of the poultry samples studied contained VRE. Out of these, 142/242 (59%) were identified as VREF, which were found nationwide in all of the participating centers. Thirty-six VRE (36/242 (15%)) were identified as *Enterococcus durans*, 34/242 (14%) as *Enterococcus hirae* and 27/242 (11%) as *E. gallinarum*. *E. faecalis* was found only three times (3/242(1%)). All VREF and VR *E. faecalis* had vancomycin MIC's of ≥ 256 µg/ml and teicoplanin MIC's of 16 - ≥ 256 µg/ml, which is indicative of the VanA phenotype. VR *E. gallinarum* had vancomycin MIC's of 8 - 16 µg/ml and teicoplanin MIC's of 1 - 3 µg/ml, the VanC phenotype. The 70 strains classified as *E. hirae* or *E. durans* had MIC's that ranged

TABLE 2 *Number and percentages of VRE isolated from 305 poultry products by 11 Health Inspectorates in The Netherlands in the period from June to September 1996.*

Region of the Food Inspection Department in The Netherlands	No. of poultry products	No. of poultry products with VRE	No. (%) of poultry products with VRE of type:				
			E. faecium	*E. durans*	*E.hirae*	*E. gallinarum*	*E.faecalis*
Den Haag	34	18	15 (44)	3 (9)	-	-	-
Maastricht	40	33	16 (40)	4 (10)	6 (15)	7 (18)	-
Alkmaar	22	17	11 (50)	2 (9)	4 (18)	-	-
Amsterdam	47	35	19 (40)	4 (9)	5 (11)	5 (11)	2 (4)
Nijmegen	25	21	6 (24)	5 (20)	3 (12)	6 (24)	1 (4)
Rotterdam	17	12	5 (29)	4 (24)	3 (18)	-	-
Leeuwarden	32	20	13 (41)	2 (6)	4 (13)	1 (3)	-
Den Bosch	16	16	10 (63)	3 (19)	3 (19)	-	-
Goes	25	25	15 (60)	5 (20)	1 (4)	4 (16)	-
Zutphen	23	21	15 (65)	1 (4)	1 (4)	4 (17)	-
Groningen	24	24	17 (71)	3 (13)	4 (17)	-	-
Total (%)*	**305**	**242 (79)**	**142 (47)**	**36 (12)**	**34 (11)**	**27 (9)**	**3 (1)**

* Percentage of all 305 poultry products

from 16 to ≥ 256 µg/ml for vancomycin and from 2 to ≥ 256 µg/ml for teicoplanin. All those VRE, except the VR *E. gallinarum*, harbored the *vanA* gene. Strains containing the *vanB* or *vanC2* gene were not found.

Pulsed Field Gel Electrophoresis

One hundred and forty two *E. faecium* and 3 *E. faecalis* isolates were analyzed by PFGE. Most of the PFGE banding patterns comprised 15 to 20 differently sized DNA fragments. The data revealed that two out of three *E. faecalis* strains were genetically identical. Both strains originated from the same geographical region. One hundred different genotypes were identified in the group of VREF poultry strains (for some examples of PFGE banding patterns, see Fig. 1). However, two genotypes of *E. faecium*, (respectively 22/142 and 14/142 (A and B; Fig. 2)), were more frequently found by ten out of eleven of the Food Inspection Services. These two genotypes could represent Dutch epidemic VREF (EVREF). When the poultry VREF strains were compared with VREF strains isolated from patients, however, no overlap in visually defined genotypes was identified by PFGE on the basis of the Tenover criteria [32]. This was essentially corroborated by Gelcompar analysis of the PFGE data of 48 strains (see Fig 3). The figure shows that the highest homology value between VRE from chicken and human is 60% (Goes 175 and Goes 178 versus 10a). Strains from the different origins present in a clustered fashion. The epidemic PFGE type A clusters at a high homology value (90% for Den Bosch 155 to Goes 84). The type that was encountered among humans relatively frequent (PFGE type M from reference [6]) clusters as well. Finally, the figure shows that chicken strains mingle with respect to the geographic origin. A total of 27 *E. gallinarum* strains could be identified on the basis of the characteristic PFGE patterns displaying DNA fragments being smaller than 200 kb only [33].

Transposon mapping by PCR and sequencing

All PCR tests for transposon mapping (Table 1) were done on a random selection of 5 human and 5 poultry strains. PCR products derived from structural Tn*1546* genes for all human and poultry strains displayed identity in size after electrophoresis. The same conclusion was reached when the intergenic *vanS-vanH* and *vanY-vanZ* regions were amplified.

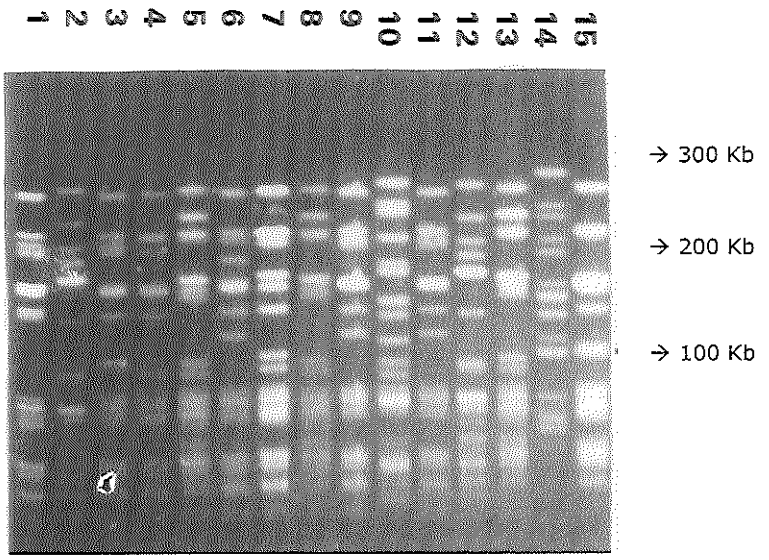


Figure 1: *PFGE patterns for 15 VREF isolated from poultry products collected by the Dutch Food Inspection Services in Zutphen, The Netherlands. Molecular lengths of markers are indicated on the right*

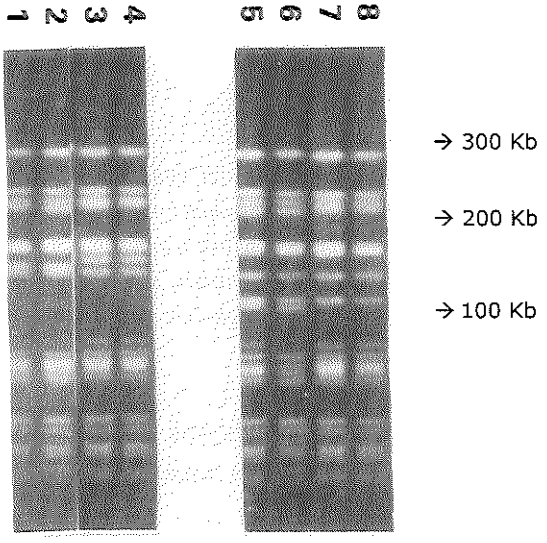


Figure 2: *PFGE patterns of two epidemic genotypes of VREF. Lanes 1 to 4, genotypeA; lanes 5 to 8 genotype B. These genotypes were frequently found by most of the Food Inspection Services. Molecular lengths of the markers are indicated on the right*

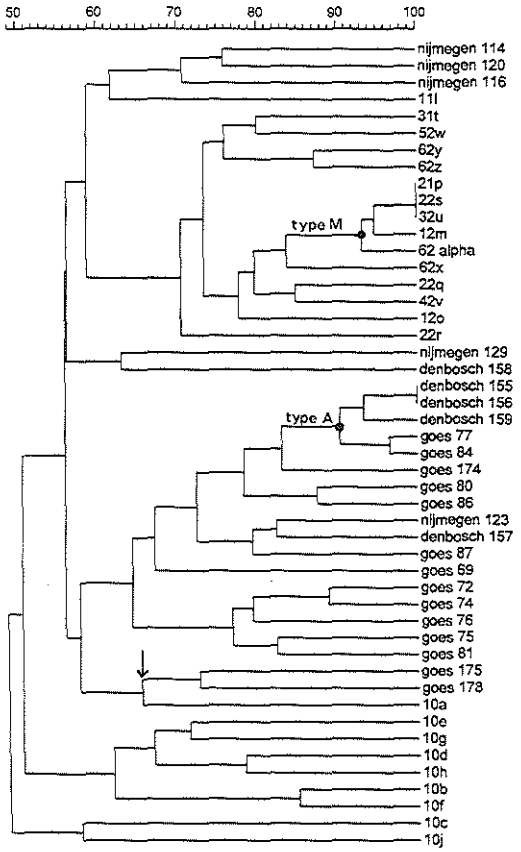


Figure 3: *Phylogentic tree constructed on the basis of several PFGE types of VREF derived from poultry products (originating from Den Bosch, Goes and Nijmegen, The Netherlands) and humans [6]. The arrow indicates the highest level of homolgy between VREF from poultry and humans (Goes 175 and Goes 178 versus 10a). Type A is the epidemic PFGE type among poultry clusters, and strains have a high homology cluster (Den Bosch 155 to Goes 84). Type m is the type that was encountered among humans relatively frequent [6]*

However, the *vanX-vanY* intergenic region of two poultry strains was ± 1300 bp in size, whereas in the other three poultry strains and the 5 human isolates the size of the PCR product was approximately 540 bp. Subsequently, all VREF (142 poultry strains and nineteen human stains) and all VR *E. faecalis* (3 poultry strains and one human strain) were analyzed with the *vanX-vanY* primer set. Both transposon types were found in all participating centers, indicating equal spread of both of these transposon types. All human strains and 83/142 (58%) of all isolated poultry VRE contained an intergenic region between *vanX* and *vanY* of approximately

540 bp. The 1300 bp fragment was not found in human strains, but in 59/142 (42%) of the poultry strains. Sequencing of the 543 bp *vanX-vanY* intergenic regions of several VREF strains from poultry as well as human origin demonstrated full sequence conservation. In case of the larger *vanX-vanY* fragment sequencing revealed the presence of *IS1216V* [34]. This element was identified before in the same location (Genbank accession number L40841 and reference [35]).

Discussion

To the best of our knowledge this is the first systematic study from continental Europe reporting a high prevalence of VRE in consumer poultry at the retail level. Glycopeptide resistance in enterococci isolated from living poultry has been associated with the use of oral glycopeptide antibiotics in animal feed [24]. High-level resistance to glycopeptides has been shown to be mediated by transferable plasmids that may harbor resistance determinants to other drugs as well [36]. Therefore, other antimicrobial agents used as feed additive in veterinary medicine may also select for vancomycin resistance. Definition of causal relationships requires detailed studies on the development and spread of antibiotic resistance in poultry farms. Comparison of resistant microorganisms derived from poultry with those derived from humans may shed light on the role of poultry as a possible reservoir of VRE.

We found that 70% of the poultry products at the retail level were contaminated with VRE containing the *vanA* gene. The majority of these VRE were *E. faecium*. A study from the UK documented that 22 out of 52 farm animals were colonized with VREF [5]. In five uncooked chicken specimens VREF was also identified. All strains possessed the *vanA* gene, which confers high-level resistance to vancomycin. A study from Manchester, United Kingdom, revealed that 90% of all uncooked chicken specimens contained VRE that were genetically distinguishable [37]. The strains differed from clinical isolates but were capable of transferring the resistance trait by conjugation experiments. Others showed that vancomycin and avoparcin resistant *E. faecium* could be detected in 5 out of 8 conventional Danish poultry farms [14]. On the other hand, among isolates from 6 ecology farms no glycopeptide resistance was observed. In Belgium, about 7% of the animals investigated for VRE carriage (horses, dogs, pigs and chicken) were colonized with VREF [13]. Interestingly, VRE have so far not been recovered from animal sources

in the United States, possibly related to the fact that glycopeptides are not licensed for use as feed additive in animal husbandry [15,16].

Twenty-seven of the poultry specimens contained *E. gallinarum*, a subspecies which is rarely found in humans neither as part of gut flora nor as clinical isolates. However, we have observed an increase in the number of *E. gallinarum* strains isolated from clinical material in our hospital since the introduction of a screen agar containing 6 mg/L vancomycin (data not shown). These observations suggest that additional research into the relevance of *E. gallinarum* as a potential pathogen in humans is needed. As enterococci are not routinely identified up to species level in many microbiology laboratories, *E. gallinarum* may well be underreported.

Two main routes of dissemination of vancomycin resistance genes can be envisaged. Firstly, resistant strains may spread in a clonal fashion from one host to the other. Secondly, the resistance determinant could be passed on to other bacterial strains through conjugation [38,39]. Two major PFGE types of VRE have been identified among poultry-derived strains. Since these types were identified in all Food Inspection Services, we are dealing with epidemic strains and not a local outbreak. Neither of these two types nor any of the other unique genotypes of VREF were found in faecal flora of patients screened for VREF carriage in The Netherlands [6]. On the basis of these results, one could reject the hypothesis that direct horizontal transmission of VRE from poultry to humans via the food chain, is a major transmission route. This is corroborated by more extensive phylogenetic analysis of the data (see figure 3). Therefore, the answer to the question on the origin of human VRE still remains obscure. Several studies suggest that high-level resistance to glycopeptides in enterococci isolated in Europe and North America be mediated by transposons similar to Tn*1546* [40]. Mapping of the transposon as present in the poultry VRE by PCR revealed the presence of two distinct *vanA* types. Length variability was found in the *vanX-vanY* regions. Among VREF from poultry, many strains including EVREF carried an intergenic region between *vanX* and *vanY* of approximately 1300 bp, not encountered in the human strains. The other poultry strains and all human strains shared an identical *vanX-vanY* intergenic region. This observation suggests that, for as yet unknown reasons, some sort of species barrier may exist for the larger transposon type or it may be limited with respect to conjugative transfer. More Dutch VRE from human should be investigated to confirm the data presented here. In contrast, another transposon type that is prevalent in many poultry strains and in all human strains may have

spread from poultry to humans via the food chain. As we studied only a limited number of structural genes and intergenic regions, further detailed analysis the vanA gene cluster is in progress to confirm that these transposons are related. Relationship between the *VanA* cluster of VRE isolated from humans and poultry was also determined by means of restriction fragment length polymorphism (RFLP) analysis of the Tn*1546*-like element. For this, several human and poultry isolates were analyzed in detail. All human isolates showed the same RFLP type as well as some poultry isolates. The other isolates from poultry contained a RFLP type, which was nearly distinct from the human RFLP type. (Work still in progress in collaboration with the National Institute of Health and Environmental Protection (RIVM, Bilthoven, The Netherlands [33]).

In conclusion, we report an extremely high prevalence of VRE in consumer poultry in The Netherlands. A high prevalence of a deviating transposon type is found in poultry VRE especially. Transmission of the resistance genes, rather than clonal dissemination of resistant microorganisms, may be the factor driving the spread of vancomycin resistance from poultry to humans. If this suggestion can be substantiated by additional research this may have major implications for the development of strategies to control the spread of glycopeptide resistance among bacterial species pathogenic to man. More information is needed to further clarify and quantify the antibiotic resistance gene transfer from bacterial isolates derived from animals or humans.

References

1. Bingen EH, Denamur E, Lambert-Zechovsky NY, Elion J. 1991. Evidence for the genetic unrelatedness of nosocomial vancomycin-resistant *Enterococcus faecium* strains in a pediatric hospital. J. Clin. Microbiol. 29:1888-1892.
2. Moulin F, Dumontier S, Saulnier P, Chachaty E, Loubeyre C, Brugieres L, Andremont A. 1996. Surveillance of intestinal colonization and infection by vancomycin resistant enterococci in hospitalized cancer patients. Clin. Microbiol. Infect. 2:192-201.
3. Jordens JZ, Bates J, Griffiths DT. 1994. Faecal carriage and nosocomial spread of vancomycin-resistant *Enterococcus faecium*. J. Antimicrob. Chemother. 35:515-528.
4. Chadwick PR, Oppenheim BA, Fox A, Woodford N, Morgenstern GR, Scarffe JH. 1996. Epidemiology of an outbreak due to glycopeptide resistant *Enterococcus faecium* on a leukemia unit. J. Hosp. Infect. 34:171-182.
5. Gordts B, Van Landuyt H, Ieven M, Vandamme P, Goossens H. 1996.Vancomycin-resistant enterococci colonizing intestinal tracts of hospalized patients. J. Clin. Microbiol. 33:2842-2846.
6. Endtz HP, Van den Braak N, Van Belkum A, Kluytmans AJW, Koeleman JGM, Spanjaard L, Voss A, Weersink AJL, Vandenbroucke-Grauls CMJE, Buiting AGM, Van Duin A, Verbrugh HA. 1997. Fecal carriage of vancomycin-resistant enterococci in hospital- and community- based patients in the Netherlands. J. Clin. Microbiol. 35:3026-3031.

7. Centers for Disease Control and Prevention. 1993. Nosocomial enterococci resistant to vancomycin in the United States, 1989-1993. MMWR 42:597-599.
8. Chow JW, Kuritza A, Schlaes DA, Green M, Sahm DF, Zervos MJ. 1993. Clonal spread of vancomycin-resistant *Enterococcus faecium* between patients in three hospitals in two states. J. Clin. Microbiol. 31:1609-1611.
9. Murray B. 1995. What can we do about vancomycin-resistant enterococci? Clin. Infect. Dis. 20:1134-1136.
10. Dunne WM, Wang W. 1997. Clonal dissemination and colony morphotype variation of vancomycin-resistant *Enterococcus faecium* isolates in metropolitan Detroit, Michigan. J. Clin. Microbiol. 35:388-392.
11. Bates J, Jordens Z, Griffiths DT. 1994. Farm animals as a putative reservoir for vancomycin resistant enterococcal infection in man. J. Antimicrob. Chemother. 34:507-516.
12. Van Belkum A, Van den Braak N, Thomassen R, Endtz H. Vancomycin-resistant enterococci in cats and dogs. Lancet 1996;348:1038-1039.
13. Devriese LA, Ieven M, Goossens H, Vandamme P Pot B, Hommez J, Haesebrouck F. 1996. Presence of vancomycin-resistant enterococci in farm and pet animals. Antimicrob. Agents Chemother. 40:2885-2887.
14. Moller Aarestrup F. 1995. Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. Microb. Drug Resist. 3:255-257.
15. Thal LA, Chow JW, Mahayni R, Bonilla H, Perri MB, Donabedian SA, Silverman J, Taber S, Zervos MJ. 1995. Characterization of antimicrobial resistance in enterococci of animal origin. Antimicrob. Agents. Chemother. 39:2112-2115.
16. Coque TM, Tomayko JF, Ricke SV, Okhyusen PC, Murray BE. 1996. Vancomycin resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob. Agents Chemother. 40:2605-2609.
17. Noble WC, Zarina Virani R, Cree RGA. 1992. Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Letters 93:195-198.
18. HICPAC. 1995. Recommendation for preventing the spread of vancomycin resistance: Recommendation of the Hospital Infection Control Practices Advisory Committee (HICPAC). Am. J. Infect. Control. 23:87-94.
19. Edmond MB, Wenzel RP, Pasculle W. 1996. Vancomycin resistant *Staphylococcus aureus*: perspectives on measures needed for control. Ann. Intern. Med 123:329-334.
20. Day M. 1997. Superbug spectre haunts Japan. New Scientist. May:521.
21. Centers for Disease Control and Prevention. 1997. *Staphylococcus aureus* with reduced susceptibility to vancomycin - United States. MMWR 46:765-766.
22. Moreira B, Boyle-Vavra S, de Jonge BLM, Daum RS. 1997. Increased production of penicillin binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 41:1788-1793.
23. Van den Boogaard AE, Stobberingh EE. 1996. Time to ban all antibiotics as animal growth-promoting agents? Lancet 31:619.
24. Bager F, Madsen M, Christensen J, Aarestrup FM. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin- resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev. Vet. Med. 31: 95-112.
25. Klare I, Heier H, Claus H, Reisbrodt R, Witte W. 1995. VanA mediated high-level glycopeptide resistance in *Enterococcus faecium* from animal husbandry. FEMS Microbiol. Letters 347:165-171.
26. Anonymous. 1987. Kanamycin aesculin azide (KAA) agar. Int. J. Food Microbiol. 5:731-734.
27. Leclerq R, Dutka-Malen S, Duval J, Courvalin P. 1992. Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum*. Antimicrob. Agents. Chemother. 36:2005-2008.
28. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495-503.
29. Dutka-Malen S, Evers S, Courvalin P. 1995. Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33:24-27.

30. Beighton D, Casewell M. Characterization of vancomycin resistance *Enterococcus faecium* strains isolated of human and poultry sources. Personal communication.
31. Miele A, Bandera M, Goldstein BP. 1995. Use of primers selective for vancomycin resistance genes to determine Van-genotype in enterococci and to study gene organization in VanA isolates. Antimicrob. Agents Chemother. 39:1772-1778.
32. 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.
33. Donadedian S, Chow JW, Shlaes DM, Green M, Zervos MJ. 1995. DNA hybridization and contour-clamped homogenous electric field electrophoresis for identification of enterococci to the species level. J. Clin. Microbiol. 33(1):141-145.
34. Willems RJL. Personal communication
35. Arthur M, Depardieu F, Gerbaud G, Galmand M, Leclercq R, Courvalin P. 1997. The *VanS* sensor negatively controls *VanR* mediated transcriptional activation of glycopeptides resistance genes of Tn*1546* and related elements in the absence of induction. J. Bact. 171(1): 97-106.
36. Leclercq R, Dutka-Malen S, Duval J, Courvalin P. 1989. Transferable vancomycin and teicoplanin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 33:10-15.
37. Chadwick PR, Woodford N, Kacmarski EB, Gray S, Barrell RA, Oppenheim BA. 1996. Glycopeptide resistant enterococci isolated from uncooked meat. J. Antimicrob Chemother. 38:908-909.
38. Salyers AA, Amabile-Cuevas CF. 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents Chemother. 41:2321-2325.
39. Arthur M, Courvalin P. 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob. Agents Chemother. 37:1563-71.
40. Van Horn KG, Gedris CA, Rodney KM. 1996. Selective isolation of vancomycin-resistant enterococci. J. Clin. Microbiol. 34:924-927.

CHAPTER 8

Random Amplification of Polymorphic DNA versus Pulsed Field Gel Electrophoresis of *Sma*I DNA Macrorestriction Fragments for Typing Strains of Vancomycin Resistant Enterococci

Nicole van den Braak, Eddy Power[1], Richard Anthony[1]
Hubert Ph. Endtz, Henri A. Verbrugh and Alex van Belkum

Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands
The Guy's and St. Thomas Hospital, London, England[1]

FEMS Microbiology Letters (2000) 192: 45-52

Abstract

Genetic typing of vancomycin resistant enterococci (VRE) can be performed using a variety of methods, but comparative analyses of the quality of these methods are still relatively scarce. We here compare random amplification of polymorphic DNA (RAPD) analysis with pulsed field gel electrophoresis (PFGE) of DNA macrorestriction fragments as examples of two of the recent and well-accepted molecular typing methods. For the latter method, empirical guidelines for the interpretation of the DNA fingerprints have been proposed in the international literature. Based on our experimental analyses, we define similar criteria for RAPD fingerprinting. A collection of 100 strains of VRE, comprising *Enterococcus faecium, E. faecalis, E. avium, E. gallinarum* and *E. casseliflavus*, was assembled. Fifty isolates were Dutch; another fifty were isolated in the United Kingdom. Strains were selected on the basis of previously determined putative identity, close relatedness or uniqueness. The strains were analyzed using well-standardized RAPD and PFGE protocols. Resulting fingerprints were interpreted with computerized methods involving band positioning and we show that typing of VRE by PFGE and RAPD generates highly congruent DNA fingerprint clustering. When the proposed international criteria for interpretation of PFGE fingerprints were applied in our case, 86% PFGE homology as discriminating value between close relatedness and uniqueness, a 75% homology cut-off for the comparison of the RAPD-generated DNA fingerprints revealed essentially identical strain clusters. As a spin-off it is revealed that strains from the different species can be efficiently discriminated, that strains from the United Kingdom and The Netherlands form separate clusters and that strains from veterinary origin can be identified separately as well.

Introduction

The need for adequate molecular typing schemes is especially relevant in cases of pathogens that either increase in clinical prevalence or gain specific features increasing their disease-causing capacity. Both facts became apparent for enterococci upon the rise in number of infections caused by these bacteria in immuno-incompetent patients and the appearance of antibiotic resistant types in the late eighties and the early nineties [1]. Several approaches for the identification below the species level were developed, among others pulsed field gel electrophoresis (PFGE) of DNA macrorestriction fragments [2]. The first study involving enterococci demonstrated the usefulness of the procedure for typing strains of *Enterococcus faecalis* [3]. Also, for *Enterococcus faecium* it was demonstrated that PFGE provided an efficient procedure for mapping genomic polymorphism in an epidemiologically concordant fashion [4].

More recently, PCR mediated procedures capable of identifying enterococcal subtypes within the different species have become available. Upon amplification of the so-called ribosomal intergenic spacer (ITS PCR) species characteristic banding patterns are generated [5]. However, the major application of PCR technology in the field of enterococcal studies is the random amplification of polymorphic DNA under relaxed hybridization conditions during the annealing phase of the PCR (RAPD analysis) [6]. This procedure generates complex mixtures of amplicons that can be translated into DNA fingerprints by simple electrophoresis in agarose gels. Comparisons with respect to the resolving power and epidemiological concordance for RAPD versus PFGE have been made and the overlap in the data suggested that RAPD analysis is well suited for epidemiological typing of enterococci [7].

Suggestions for the adequate interpretation of data provided by PFGE have been published in recent literature [8]. Although these suggestions provide an adequate framework for determining genetic relatedness in case of a local outbreak situation, little is known on the molecular basis of the changes that can be observed in the banding patterns. In addition, the two multicenter studies performed to date on *Staphylococcus aureus* strains show a disappointing degree of reproducibility between centers (see [9] and references therein). Because of these inconsistencies, we became interested in the establishment of inter-strain relatedness of vancomycin resistant enterococci (VRE) by multiple DNA mediated

technologies in order to verify the guidelines brought forward for PFGE [8] while at the same time trying to establish similar frameworks for RAPD typing data [10]. Hundred VRE strains from the United Kingdom and The Netherlands were combined in a single collection and for all strains both RAPD and PFGE was performed. Subsequently, the banding patterns were interpreted and maximizing the overlap in types obtained by both techniques helped in the formulation of guidelines for the interpretation of banding patterns and inferring reliable inter-strain relatedness.

Materials and Methods

Bacterial strains

A collection of 100 VRE strains was analyzed in the present study. Fifty VRE strains isolated in The Netherlands and fifty VRE strains isolated in the United Kingdom were included. This collection of VRE included the species *E. faecium, E. faecalis, Enterococcus avium, Enterococcus gallinarum and Enterococcus casseliflavus* isolated from diverse sources (Table 1). All enterococci were identified to the species level on the basis of colony morphology, Gram stain, pyrase and catalase testing, pigment production, the presence of the Lancefield Group D antigen and Rapid ID32 Strep (bioMerieux, 's Hertogenbosch, The Netherlands) or API 20STREP system (bioMerieux, Basingstoke, UK). English strains were selected on the basis of earlier RAPD analyses as performed in the St. Thomas' Hospital, London, United Kingdom [12]. Dutch strains were chosen on the basis of former PFGE-determined genotypes as performed at the EMCR, Rotterdam, The Netherlands (e.g. [11]). Twenty-four out of fifty Dutch VRE (48%) were isolated from poultry products and 26/50 (52%) were gathered from humans. Each collection contained presumptively unique, related and identical genotypes.

Pulsed field gel electrophoresis

PFGE was used as "gold" standard and performed for all strains at the EMCR, Rotterdam, The Netherlands. Samples of genomic DNA extracted from the strains were digested with *Sma*I (Boehringer Mannheim, Mannheim, Germany) and compared by PFGE as described previously [11].

Table 1: *Dutch and UK VRE strains used and their origin*

Species	Strain	Source of Isolation
E. faecium	d2, d3, d5, d11, d12, d14, d19, d20, d21, d24, d25 d26, d28, d31, d36, d38, d39, d40, d42, d43, d45, d46	poultry
	d23, e3, e5, e6, e9, e7, e8, e12, e17, e18, e21, e23, e24, e25, e26, e29, e38, e42, e43, e46, e48, e50	urine
	d1, d9, d10, d17, d27, d29, d30, d32, d35, d37, d41 d49, d50	rectum
	e16, e22, e33, e35, e47	stool
	d4, e37	wound
	d13	ascites
	d22, e36	blood
	d47	bile
	e4	wound-line
	e27	central line tip
E. faecalis	d6	ascites
	d15, d33	poultry
	e10, e13	stool
	e11	tip catheter
	e19	central line tip
	e20, e28	throat swab
	e32, e40	urine
	e34	vascath tip
	e44	exit site
	e45	unknown
E. avium	e14	CAPD
	e15, e41	blood
	e30	unknown
E. gallinarum	d7, d16, d18, d48	rectum
	e1, e2, e49	blood
E. casseliflavus	d8, d34, d44	rectum
	e31	blood
	e39	unknown

NB The source of the isolates is indicated with respect to anatomical or veterinary niche. Dutch strains share a prefixed d in the code, the UK strain codes start with e. The *E. gallinarum* and *E. casseliflavus* strains derived from a surveillance study performed in cohorts of volunteers and vegetarians. Additional data on the Dutch strains can be derived from references [11, 20, 21], details concerning the UK isolates can be derived from [12]. CAPD: chronic ambulatory peritoneal dialysis.

Electrophoresis (1% SeaKem agarose in 0.5x TBE) was performed using a BioRad CHEF mapper, programmed in the auto-algorithm mode (block 1: runtime 8 hours; switchtime: 0.5-15 seconds and block 2: runtime 10 hours; switchtime 15-30 seconds). The gel was stained with ethidium-bromide for 15 minutes and then destained in distilled water for 1 hour before photography under UV irradiation. The PFGE patterns were interpreted according to Tenover *et al* [8]. Isolates were considered identical and representative of a single strain if they showed identical PFGE patterns. Isolates that differed by 1-3 bands, consistent with a single differentiating genetic event, were assigned a numbered subtype. Four or more band-differences between two strains defined different genotypes. Data obtained for all VRE were studied in more detail using Gelcompar software (Applied Maths, Gent, Belgium). The PFGE patterns were scanned and Dice analysis of peak positions was executed. The Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was applied and the bandwidth tolerance was set critically at 1.2%.

DNA extraction and random amplified polymorphic DNA (RAPD) analysis

DNA extraction and RAPD were performed for all strains in England (St. Thomas' Hospital, London). DNA was isolated as described previously [12]. RAPD was performed in volumes of 50 µl containing 1x DNaZyme DNA polymerase buffer (Flowgen, Leicestershire, United Kingdom), 200 µM of each deoxyribonucleotide triphosphate, 5 µl of template DNA (equaling approximately 50 ng), and 1 U DNaZyme polymerase. Two different primers were used in separate assays at a concentration of 1 µM. The sequences were TGCTCTGCCC (AB106) and GTAGACCCGT (AB111). The reaction was overlaid with 50 µl of paraffin oil. Amplification of DNA was performed in a Techne PHC-3 model thermal cycler (Techne Ltd, Duxford, Cambridge, United Kingdom) using 35 cycles of 30 sec at 95° C, 30s at 34° C and 1 min at 72° C. Banding patterns were visualized under UV light, after electrophoresis on a 2% agarose gel and ethidium bromide staining. Two independent researchers categorized banding patterns, and (sub) types were assigned on the basis of band differences. Data obtained for all VRE were studied in more detail using Gelcompar software. Dice analysis of peak positions was executed, UPGMA was applied and the bandwidth tolerance was set critically at 1.2%.

Results

PFGE analysis

Most of the PFGE patterns comprised of 15 to 20 differently sized DNA fragments ranging from approximately 50 Kb to 450 Kb in size (Figure 1). Overall, 11 clusters were identified, a cluster being a set of strains ($n \geq 2$) showing more than 86% homology. Among VRE isolated in The Netherlands, 31 different genotypes were obtained by PFGE analysis, including three clonally related clusters of strains (cluster *I* - *III* (n=22 strains)), according to the Gelcompar analysis based on a homology of >86%. Strains of cluster *I* were completely identical. Cluster *II* and *III* contained isolates that showed identical or almost identical (up to three-band difference) PFGE-derived patterns. PFGE data of the English VRE revealed that 19 different genotypes were identified according to the same interpretation guidelines. Strain e16, e20 and e22 were untypable, probably due to endogenous endonuclease activity. Eight clusters containing multiple isolates (cluster *IV* - *XI* (n=35)) were identified in addition, whereas 11 isolates had unique PFGE-derived patterns. Ten out of 15 strains of cluster *IV* were PFGE identical and the other five strains differed in 1-3 bands and were classified as closely related isolates. There was no apparent overlap between English and Dutch VRE PFGE genotypes examined in this study. The maximum level of homology between these clusters was 80 % (English VRE strains in cluster X (e1, e2 and e49) and Dutch VRE strain 6).

Multiple *Enterococcus* species were included in this study. Characteristic PFGE patterns with all fragments smaller than 200 Kb was found for 6/7 *E. gallinarum* strains (Figure 1). PFGE analysis of *E. faecalis* strains revealed some specific fragments of more than 400 Kb in size. Nine out of thirteen *E. faecalis* strains were in cluster *V, VI* and *XI* and the other four strains were unique genotypes that scattered throughout the dendrogram. Most of the species *E. gallinarium, E. avium* and *E. casseliflavus* were found in one single branch with a homology between 52% and 60%. The Dutch VRE group contained 24 poultry isolates, 12 of them had unique genotypes and the other 12 were placed in clusters II and III. No close homology was found between VRE isolated from humans and poultry.

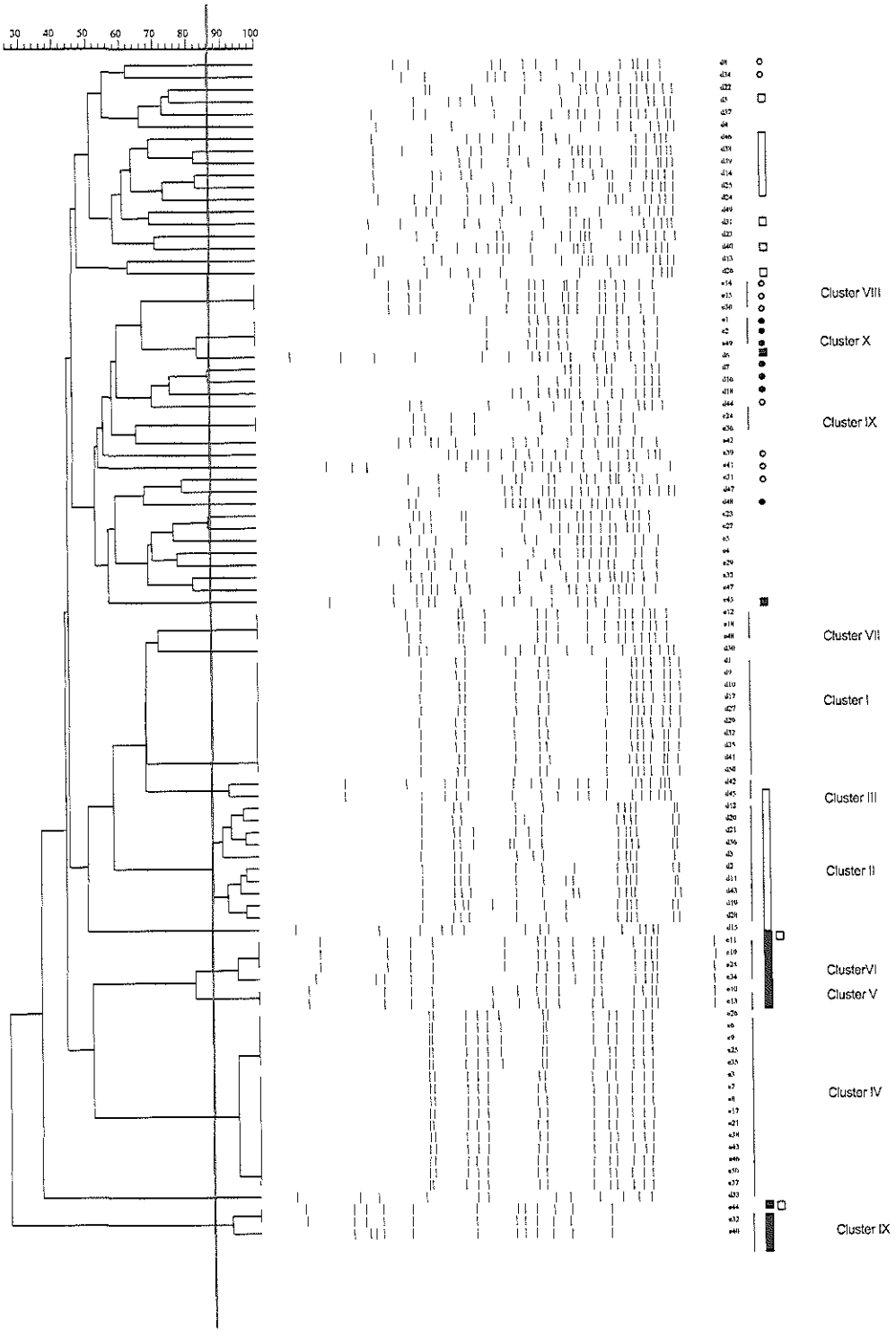

30
40
50
60
70
80
90
100
Cluster VIII
Cluster X
Cluster IX
Cluster VII
Cluster I
Cluster III
Cluster II
ClusterVI
Cluster V
Cluster IV
Cluster IX

Figure 1: *Dendrogram showing the clustering of VRE strains isolated in The Netherlands (d prefixed to the identification number) or in the United Kingdom (e prefix). The strains were analyzed with PFGE and clustering took place on the basis of a homology of 86% (vertical line, the homology percentage bar is presented on top). Clusters are identified by Roman numbering. All strains are described in Table 1. Different species are marked in the dendrogram. Most strains are E. faecium; the following characters are used to identify strains belonging to other species:*

□: VRE from poultry origin; ○ : *E. casseliflavus*; ● : *E. gallinarum*; ◌ : *E. avium*; ■: *E. faecalis*.

RAPD analysis

Banding patterns obtained from RAPD analysis comprised of 8 to 14 differently sized DNA fragments (Figure 2). Overall, 18 clusters were identified. The Dutch VRE showed 7 clusters (cluster A-G (n=34)) and 12 strains had unique RAPD patterns, based on a homology of < 75% upon Gelcompar analysis. Fourteen different genotypes were analyzed among the English VRE RAPD-derived banding patterns. Among these genotypes 10 types were found on multiple occasions (cluster J-Q (n=26)) and 8 strains had unique RAPD patterns. Interestingly, clusters R and I consisted of both Dutch and English VRE strains

Analyzing the different species, we found that 11/13 *E. faecalis* strains were clustered, the other two strains displaying unique genotypes found in the same branch as the other *E. faecalis* strains (homology 55%). Most of the species *E. gallinarium, E. avium* and *E. casseliflavus* were found in two branches with a homology of 40% and 45%. Twenty-four isolates of Dutch poultry-derived VRE were included. Seven of these strains had unique genotypes, whereas the other 17 isolates clustered. Both clusters included strains from human origin as well.

RAPD versus PFGE

The comparison between PFGE and RAPD data gathered for Dutch and UK VRE can be deduced from Figures 1 and 2. Overall, PFGE typing resulted in 11 clusters (cluster *I-XI*) containing genetically related or identical strains versus 18 clusters (cluster A-R) using the RAPD typing method. Some of these clusters are identical (I = A; V = J; VI = J; VII = K; VIII = L; X = N). This concerns 25 strains. Several strains (n = 40) are identified as unique genotypes by both techniques. For clusters II and III, relatively small numbers of strains do not seem to belong to these clusters on the basis of RAPD. In case of cluster IV, 3/15 strains are deemed unique by RAPD analysis.

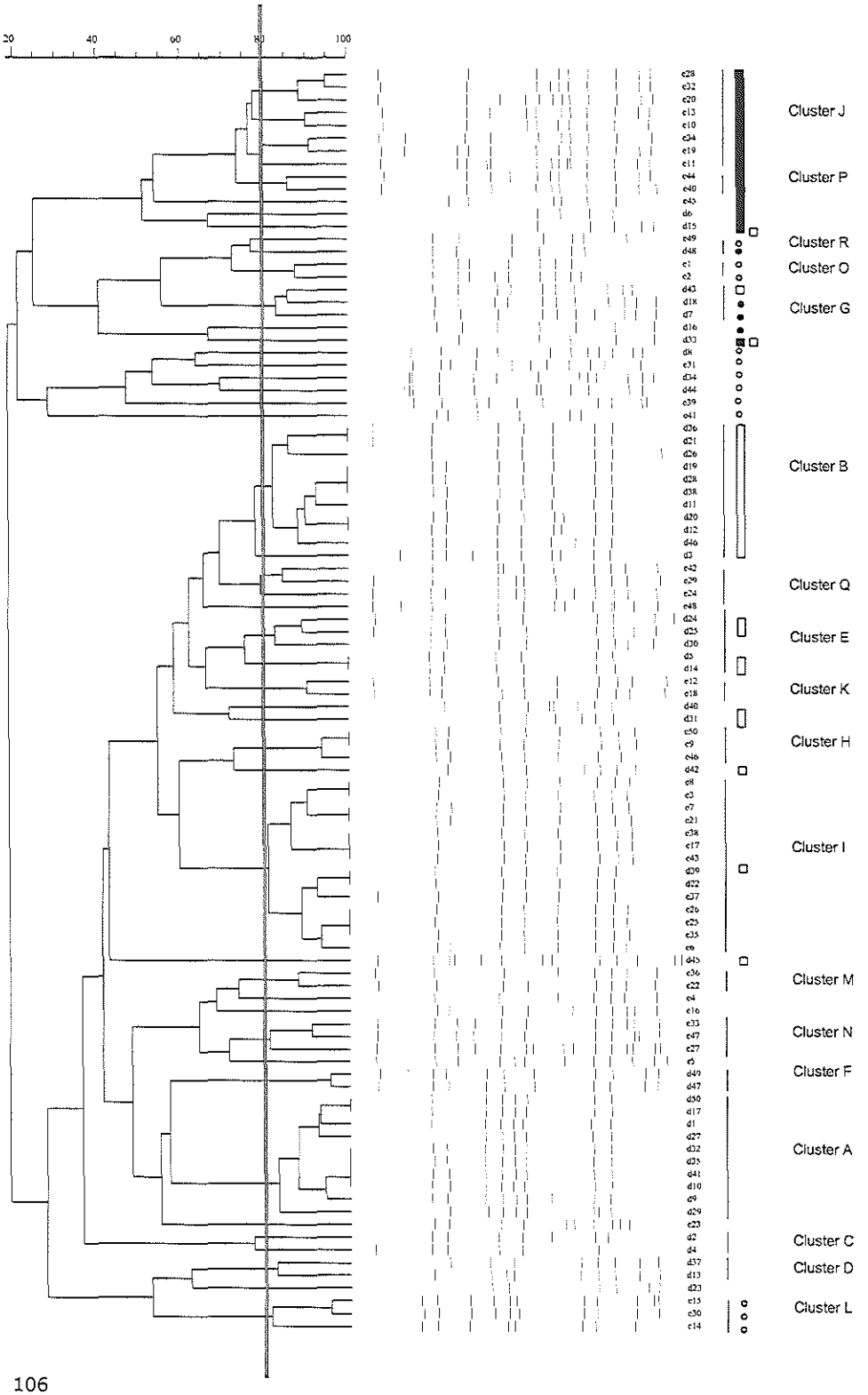

20
40
60
80
100
Cluster J
Cluster P
Cluster R
Cluster O
Cluster G
Cluster B
Cluster Q
Cluster E
Cluster K
Cluster H
Cluster I
Cluster M
Cluster N
Cluster F
Cluster A
Cluster C
Cluster D
Cluster L

Figure 2: *Dendrogram showing the clustering of VRE strains isolated in The Netherlands (d prefixed to the identification number) or in the United Kingdom (e prefix). The strains were analyzed with random amplification of polymorphic DNA analysis and clustering took place on the basis of a homology of 75% (vertical line, the homology percentage bar is presented on top). Clusters are identified by capital letter codes, All strains are described in Table 1, identification symbols are as described in the legend to Figure 1.*

Based on this general survey, 89 of 100 strains are categorized in an identical fashion both by PFGE and RAPD. RAPD group B, for instance, contained 8/10 strains of PFGE group II and 2 PFGE-unique strains. The other strains of PFGE group II were assorted in RAPD group C and H, clustering with some unique PFGE genotypes. RAPD group D, E, F, and G comprised two VRE strains identified as unique genotypes applying the PFGE method. More clustering of PFGE-unique genotypes was found in RAPD group G and N. We also noticed that some PFGE clusters were resolved by RAPD typing. VRE of PFGE cluster III and 1/3 strains of cluster VIII gave rise to multiple unique RAPD types. Comparison of PFGE and RAPD data supports the conclusion that PFGE deduced relatedness among strains is corroborated by RAPD and *vice versa*. If published interpretation guidelines for PFGE are implemented [8]; a homology setting of 75% for interpretation of the RAPD data gives optimal overlap between the data sets. Lowering or increasing this percentage leads to diminished concordance between data sets (results not shown).

Discussion

Molecular typing studies need to be performed using a standard protocol in order to increase inter-laboratory reproducibility. For PFGE this level of laboratory standardization has not yet been achieved [9]. However, it has been evident for some years that PFGE is a reliable technique for helping solve epidemiological problems. Lack of intercenter reproducibility of RAPD assays has also been identified before, but in a single institution this methodology can be standardized to a satisfactory level [13]. This gives us the confidence that both the RAPD and the PFGE tests performed during the present study give an appropriate index of genetic diversity among the strains tested.

Recently, the use of repetitive sequence-based PCR and PFGE were compared for typing of *Enterococcus faecalis* at the sub-species level. This study concluded that PFGE is the more reliable technique as the interpretation of the data and the

assay reproducibility is clearly superior to those of repeat PCR [14]. In the present study we analyzed two geographically diverse VRE collections (n=100) performing both PFGE ("gold standard") and RAPD genotyping methods. Using the PFGE method, 11 clusters could be identified among the Dutch and English VRE strains. In comparison, RAPD analysis revealed 18 VRE clusters. PFGE-unique VRE types that clustered when RAPD analysis was performed produced most of the discrepancies. RAPD analysis of these VRE strains showed no relationship with VRE strains that clustered with PFGE. These phenomena have been described before for VRE and other organisms [7, 10, 15].

The PFGE interpretation guidelines we used in this study were recommended by Tenover *et al* [8]. These guidelines are comparable to a Gelcompar setting of a homology of >86% in PFGE banding patterns comparison. However, data obtained for two strains (d7 and d16) were not in concordance with these interpretation guidelines. Strain d7 and d16 revealed a 3-bands difference and showed a homology of 85% only. In contrast, strain e23 and e27 showed a common homology of 85% as well, while differing in 4 bands upon PFGE typing. These marginal findings of two different interpretations of Gelcompar analysis can be explained on the basis of relatively small numbers of bands revealed after PFGE analysis. Fingerprints for strain d7 and d16 comprised 10 to 12 bands, whereas the average VRE PFGE patterns showed 15-20 bands.

The collection of VRE isolated in The Netherlands contained strains from different clinical and environmental sources. PFGE did not reveal overlap between human strains isolated in The Netherlands and England versus VRE from poultry origin. These findings are in concordance with a former study we performed in The Netherlands [11]. However, several studies in Europe showed some indistinguishable VRE strains isolated from humans and animals [16, 17]. In contrast, the RAPD analysis presented here revealed 17 poultry strains that showed homology with VRE isolated in humans. This phenomenon can be related to a presumptively lower discriminatory power of RAPD analysis. A possible explanation of our results would be that transmission of resistant strains is not the exclusive way by which resistance is spread. Interestingly, various studies suggested that horizontal transmission of Tn1546 transposon might be an alternative determinant factor driving the spread of vancomycin resistance [18]. It remains surprising to see that intermingling hardly takes place, since several studies in the United States showed strain dissemination of enterococci between

geographically distinct hospitals (e.g. [19]). Further studies should also take Tn1546 diversity into account.

In conclusion, PFGE and RAPD performed on diverse strains of VRE are largely concordant in outcome. Although exceptions occur, clustering leads to homologous identifications. This indicates that both methods can be used for adequate molecular typing and that data interpretation guidelines for RAPD can be set at a homology value of approximately of 75% which is lower than the suggested value for PFGE. However, in case of clonal outbreaks we suggest to confirm strain relatedness with PFGE. The current analysis revealed that UK strains, even the epidemic ones, appear to be generally distinct from Dutch strains. Future studies should include larger numbers of strains and should be focussed on developing a robust, multicenter reproducible typing protocol for PFGE.

References

1. Murray BE: The life and times of the enterococcus. Clin. Microbiol. Rev. (1990) 3: 46-65.
2. Smith CL, Cantor CR: Purification, specific fragmentation and separation of large DNA molecules. Methods Enzymol. (1987) 155:499-567.
3. Murray BE, Singh KV, Heath, JD, Sharma BR, Weinstock GM: Comparison of genomic DNA of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J. Clin. Microbiol. (1990) 28 2059-2063.
4. Miranda AG, Singh KV, Murray BE: DNA fingerprinting of *Enterococcus faecium* by pulsed field gel electrophoresis may be a useful epidemiologic tool. J. Clin. Microbiol. (1991) 29:2752-2757.
5. Tyrrell G, Bethune RN, Willey B, Low DE: Species identification of enterococci via intergenic ribosomal PCR. J. Clin. Microbiol. (1997) 35:1054-1060.
6. Van Belkum A: DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev. (1994) 7:174-184.
7. Barbier N, Saulnier P, Chachaty E, Dumontier S, Andremont A: Random amplified polymorphic DNA typing versus pulsed field gel electrophoresis for epidemiological typing of vancomycin-resistant enterococci. J. Clin. Microbiol. (1996) 34:1096-1099.
8. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B: Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. (1995) 33:2233-2239.
9. Van Belkum A, van Leeuwen W, Kaufman ME, Cookson B, Forey F, Etienne J, Goering R, Grubb W, Tassios P, Legakis N, Morvan A, El Solh N, De Rijck R, Struelens M, Tenover F, Salmenlinna S, Vuopio-Varkila J, Talens A, Witte W, Verbrugh HA: Assessment of resolution and intercenter reproducibility of *Staphylococcus aureus* genotyping by pulsed field gel electrophoresis of *Sma*I macrorestriction fragments: a multicenter study. J. Clin. Microbiol. (1998) 36:1653-1659.
10. Renders N, Romling U, Verbrugh HA, van Belkum A: Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA analysis or pulsed field gel electrophoresis of DNA macrorestriction fragments. J. Clin. Microbiol. (1997) 34:3190-3195.
11. Van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz, HPh: Molecular

characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in The Netherlands. J. Clin. Microbiol. (1998) 36:1927-1936.

12. Issack MI, Power EGM, French GL: Investigation of an outbreak of vancomycin-resistant *Enterococcus faecium* by random amplified polymorphic DNA (RAPD) assay. J. Hosp. Infect. (1996) 33:101-200.
13. Van Belkum A, Kluijtmans J, van Leeuwen W, Bax R, Quint W, Peters E, Fluit A, van den Brule A, Koeleman H, Melchers W, Meis J, Vaneechoutte M, Elaichouni A, Moonens F, Maes N, Struelens M, Tenover FC, Verbrugh HA: Multicenter evaluation of polymerase chain reaction mediated DNA fingerprinting for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. (1995) 33:1537-1547.
14. Malathum K, Singh KV, Weinstock GM, Murray BE: Repetitive sequenced based PCR versus pulsed field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. J. Clin. Microbiol. (1998) 36:211-215.
15. Saulnier P, Bourneix C, Prevost G, Andremont A: Random amplified polymorhic DNA assay is less discriminant than pulsed-field gel electrophorisis for typing strains of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. (1993) 31:982-985.
16. Descheemaeker PRM, Chapelle S, Devriese LA, Butaye P, Vandamme P, Goossens H: Comparison of glycopeptide resistant *Enterococcus faecium* isolates and glycopeptide resistance genes of human and animal origin. Antimicrob. Agents Chemotherp. (1999) 43:2032-2037.
17. Stobbering E, van den Bogaard A, London N, Driessen C, Top J, Willems R: Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughters and (sub)urban residents in the south of The Netherlands: evidence for transmission of vancomycin resistance from animals to human? Antimicrob. Agents Chemotherp. (1999) 43:2215-2221.
18. Willems RJL, Top J, van den Braak N, van Belkum A, Mevius DJ, Hendriks G, Santen-Verheuvel M, van Embden JDA: Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. Antimicrob. Agents Chemother. (1999) 43:483-491.
19. Sader HS, Pfaller MA, Tenover FC, Hollis RJ, Jones RN: Evaluation of characterization of multi-resistant *Enterococcus faecium* from 12 U.S. medical centers. J. Clin. Micrbiol. (1994) 32:2840-2842.
20. Endtz HPh, Van den Braak N, Van Belkum A, Kluytmans AJW, Koeleman JGM, Spanjaard L , Voss A, Weersink AJL, Vandenbroucke-Grauls CMJE, Buiting AGM, Van Duin A, Verbrugh HA: Fecal carriage of vancomycin-resistant enterococci in hospital- and community- based patients in the Netherlands. J. Clin. Microbiol. (1997) 35:3026-3030.
21. Van den Braak N, Kreft D, van Belkum A, Verbrugh HA, Endtz HPh: Vancomycin-resistant enterococci in vegeterians. Lancet. (1997) 350:146-147.

CHAPTER 9

Molecular Diversity and Evolutionary Relationships of T*n1546*-Like Elements in Enterococci from Humans and Animals

Rob J. L. Willems[1], Janetta Top[1], Nicole van den Braak[2], Alex van Belkum[2], Dik J. Mevius[3], Giel Hendriks[1], Marga van Santen-Verheuvel[1] and Jan D. A. van Embden[1]

National Institute of Public Health and the Environment, Bilthoven, The Netherlands[1]
Erasmus university Medical Center Rotterdam, Rotterdam, The Netherlands[2]
DLO-Institute for Animal Science and Health, Lelystad, The Netherlands[3]

Antimicrobial Agents and Chemotherapy (1999) 43: 483-491

Abstract

We report on a detailed study on the molecular diversity and evolutionary relationships of T*n1546*-like elements in vancomycin-resistant enterococci (VRE) from humans and animals. Restriction fragment length polymorphism (RFLP) analysis of the VanA transposon of 97 VRE revealed seven different T*n1546* types. Subsequent sequencing of the complete VanA transposons of 13 VRE isolates representing the seven RFLP types followed by sequencing of the identified polymorphic regions in 84 other VanA transposons resulted in the identification of 22 different T*n1546* derivatives. Differences between the T*n1546* types included point mutations in *orf*1, *van*S, *van*A, *van*X, and *van*Y. Moreover, insertions of an IS*1216*V-IS3-like element in *orf*1, of IS*1251* in the *vanS-vanH* intergenic region, and of IS*1216V* in the *vanX-vanY* intergenic region were found. The presence of insertion sequence elements was often associated with deletions in T*n1546*. Identical T*n1546* types were found among isolates from humans and farm animals in The Netherlands, suggesting the sharing of a common vancomycin resistance gene pool. Application of the genetic analysis of T*n1546* to VRE isolates causing infections in hospitals in Oxford, United Kingdom, and Chicago, Ill., suggested the possibility of the horizontal transmission of the vancomycin resistance transposon. The genetic diversity in T*n1546* combined with epidemiological data suggests that the DNA polymorphism among T*n1546* variants can successfully be exploited for the tracing of the routes of transmission of vancomycin resistance genes.

Introduction

In recent years, the nosocomial prevalence of infections caused by vancomycin-resistant enterococci (VRE) has in-creased significantly in the United States [1, 2], while virtually no VRE have been found in the gut flora of healthy people [3]. The epidemiology of VRE in Europe differs from that in the United States. The prevalence of VRE in Europe is low among strains causing hospital-associated infections [4-6], while VanA-positive enterococci can easily be detected outside the hospital in several European countries [4, 7-12]. A possible source of VRE is the food chain since VRE have been isolated from farm animals and animal products in several European countries [10, 11, 13-20]. It has been suggested that the use of the antibiotic avoparcin as a feed additive in animal husbandry in numerous European countries has resulted in the selection of vancomycin resistance in strains from farm animals [14, 21, 22]. This is consistent with the lack of non-hospital-associated VRE in the United States, where the use of avoparcin has not been permitted [3].

Although resistance to glycopeptides has spread primarily in enterococci, *van*A- and *van*B-related genes were recently isolated from various other gram-positive bacteria like *Arcanobacterium, haemolyticum* [23], *Oerskovia turbata* [23], *Streptococcus bovis* [24], and *Bacillus circulans* [25]. Vancomycin resistance may disseminate to other pathogens, such as methicillin-resistant *Staphylococcus aureus* strains, which would result in a highly dangerous pathogen that could cause an infection that would be difficult to treat with currently available antibiotics. Indeed, conjugative transfer of glycopeptide resistance from *Enterococcus faecalis* to *S. aureus* has been reported under laboratory conditions [26]. The possibility that such a transfer will eventually occur in nature stresses the need to limit the spread of VRE and to gain insight into the factors that contribute to the selection of VRE and the routes of dissemination.

The genes encoding the VanA and VanB types of vancomycin resistance are located on mobile DNA elements. Therefore, the horizontal transfer of resistance genes among enterococci may have a more significant impact on the dissemination of vancomycin resistance than the clonal spread of resistant enterococci. The isolation of genetically unrelated VREs during well-documented nosocomial outbreaks suggests such a mechanism [27-31]. Thus, direct

comparison of the vancomycin resistance determinants may provide additional insight into the epidemiology of vancomycin resistance. The *vanA* gene is the most frequently encountered gene among isolates causing VRE infections in humans [4, 5, 32-34]. This gene is part of the transposable element T*n1546*, which was first characterized in 1993 by Arthur et al. [35]. Genetic heterogeneity in T*n1546*-related elements has been documented previously [10, 19, 35-43]. The polymorphisms described so far have included insertion of the insertion sequence (IS) elements IS*1216*V, IS*1251*, IS*1476*, and IS*1542* and deletions at both the left *(orf1* side) and right *(vanZ* side) ends of the transposon that includes the *orf1* and *vanZ* genes. Recently, a point mutation in the *vanX* gene has been described [10, 39].

The aim of the present study was to perform a detailed molecular characterization of the DNA polymorphisms in the *vanA* gene cluster originating from human and animal sources. By means of restriction fragment length polymorphism (RFLP) analysis and DNA sequencing, 22 different VanA transposon types among 97 VRE strains were identified. Differences included point mutations in the *orf*1, *van*A, *van*X, and *vanY* genes, the presence of the IS elements IS*1251* and IS*1216*V, and deletions associated with IS insertions. Indistinguishable T*n1546*-like elements were found among enterococci isolated from human and animal sources, suggesting the existence of a common vancomycin resistance gene pool.

Materials and Methods

Bacterial strains

The VRE used in this study are listed in Table 1. Stool samples from nonhospitalized individuals were collected and cultured in kanamycin-esculin-azide enrichment broth (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 6 mg of vancomycin per ml. Bacteria from tubes whose contents turned black after 1 or 2 days of incubation at 37° C were subcultured onto Slanetz and Bartley agar (Oxoid Ltd.) supplemented with 6 mg of vancomycin per ml. VRE were identified to the species level and were tested for the presence of the vanA gene by means of a PCR described by Dutka-Malen et al. [44]. Fecal samples from veal calves were examined as described above. Dutch clinical isolates (isolates 11 to 21), pig isolates (isolates 27 to 37), and chicken isolates (isolates

38 to 45) have been described previously [11, 19, 33], as have the isolates from The United Kingdom (isolates 46 to 87) [15, 34] and the United States (isolates 88 to 97) [45, 46].

Susceptibility testing

MICs were determined by the agar dilution method on Mueller-Hinton II agar plates (BBL, Becton Dickinson, Cockeysville, Md.). Inocula (approximately 10 8 CFU/ml) were prepared from overnight cultures on Columbia agar plates supplemented with defribrinated horse blood (Oxoid Ltd.). The antimicrobial agents tested were vancomycin (Eli Lilly, Indianapolis, Ind.), teicoplanin (Hoechst Marion Roussel Inc., Frankfurt, Germany), and avoparcin (Roche Pharmaceuticals, Basel, Switzerland).

Pulsed Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) analysis was performed as described previously [19]. The banding patterns were interpreted as described by Tenover et al. [47], and the different types were identified by capital-letter codes.

RFLP analysis

Genomic DNAs from all VRE were isolated by a modification of the initial steps of the method described by Ausubel et al. [48]. The bacterial pellets were suspended in 557 µl of 10 mM Tris–1 mM EDTA, and 10 µl of a 50-mg/ml solution of egg white lysozyme (Boehringer Mannheim, Mannheim, Germany) was added. After incubation for 15 min at 37° C, the bacteria were lysed by the addition of 30 µl of 10% sodium dodecyl sulfate and 3 µl of a 20 mg/ml proteinase K (Merck, Darmstadt, Germany) solution. Subsequently, the protocol described by Ausubel et al. [46] was used. Chromosomal DNA preparations were digested with *Hae*III and *Xba*I (Boehringer Mannheim), respectively, separated by agarose gel electrophoresis (1.5% agarose gels), transferred onto a Hybond N 1 nylon membrane (Nycomed Amersham plc, Buckinghamshire, United Kingdom) with a vacuum blotting system (Millipore, Bedford, Mass.), and subsequently hybridized with internal T*n1546* PCR fragments (probes 1, 2, 3, and 4 generated with primers 22.F-1913.R, 3514.F-5374.R, 5235.F-7035.R, and 8544.F-10716.R,

Table 1: *Enterococcal isolates used in this study*

Strain No.	Strain	Enterococcal species	Source	Country	Tn*1546* -types	Reference
1	9600188	*E. faecium*	Human stool	NL	A2	This study
2	9600205	*E. faecalis*	Human stool	NL	A1	This study
3	9600220	*E. faecium*	Human stool	NL	A1 *b*	This study
4	9600224	*E. faecium*	Human stool	NL	A2	This study
5	9600253	*E. faecium*	Human stool	NL	A2	This study
6	9600266	*E. faecium*	Human stool	NL	A2	This study
7	9600276	*E. faecium*	Human stool	NL	A1	This study
8	9600291	*E. faecium*	Human stool	NL	A1	This study
9	9700196	*E. faecium*	Human stool	NL	A1	This study
10	9700228	*E. faecium*	Human stool	NL	A2	This study
11	22-R	*E. faecium*	Human stool	NL	A2	4
12	10-A	*E. faecium*	Human wound	NL	A1	4
13	10-B	*E. faecium*	Human ascites	NL	A2	4
14	10-C	*E. faecium*	Human blood	NL	A2	4
15	10-D	*E. faecium*	Human urine	NL	A2	4
16	10-G	*E. faecium*	Human bile	NL	A2	4
17	10-H	*E. faecium*	Human blood	NL	A2 *b*	4
18	10-J	*E. faecalis*	Human ascites	NL	A1	4
19	1245964	*E. faecium*	Human urine	NL	A2	This study
20	2074639	*E. faecium*	Human ascites	NL	A2	This study
21	4252948	*E. faecium*	Human ascites	NL	E6	This study
22	1-A2	*E. gallinarum*	Veal calf	NL	A1 *b*	This study
23	1-A6	*E. flavescens*	Veal calf	NL	A3 *b*	This study
24	1-A8	*E. faecalis*	Veal calf	NL	B1 *b*	This study
25	1-A10	*E. avium*	Veal calf	NL	A1	This study
26	1-A11	*E. faecium*	Veal calf	NL	A4 *b*	This study
27	A2	*E. faecium*	Pig	NL	A2	11
28	A4	*E. faecium*	Pig	NL	A2	11
29	A16	*E. faecium*	Pig	NL	A2	11
30	B9	*E. faecium*	Pig	NL	A2	11
31	B37	*E. faecium*	Pig	NL	A2	11
32	M4	*E. faecium*	Pig	NL	A2	11
33	M7	*E. faecium*	Pig	NL	A2	11
34	M11	*E. faecium*	Pig	NL	A2	11
35	O12	*E. faecium*	Pig	NL	A2	11
36	O118	*E. faecium*	Pig	NL	A2	11
37	O122	*E. faecium*	Pig	NL	A2	11
38	chicken 2	*E. faecium*	Chicken	NL	E3	19
39	chicken 3	*E. faecium*	Chicken	NL	A1	19
40	chicken 43	*E. faecium*	Chicken	NL	A2	19
41	chicken 48	*E. faecium*	Chicken	NL	B2	19
42	chicken 57	*E. faecium*	Chicken	NL	E2 *b*	19
43	chicken 59	*E. faecium*	Chicken	NL	A1	19
44	chicken 69	*E. faecium*	Chicken	NL	E5	19
45	chicken 72	*E. faecium*	Chicken	NL	A1	19
46	58538 (GP)	*E. faecium*	Human stool	UK	E2	34
47	61741 (GP3)	*E. faecium*	Human stool	UK	A1	15
48	55859 (pat. 12)	*E. faecium*	Human stool	UK	D1 *b*	34
49	59479	*E. faecium*	Human stool	UK	D1	34
50	60761	*E. faecium*	Human stool	UK	D1	34
51	63910	*E. faecium*	Human stool	UK	C *b*	34
52	67668	*E. faecium*	Human stool	UK	A1	34
53	53864 (pat. 3)	*E. faecium*	Human stool	UK	D1	34
54	77364 (pat. 10)	*E. faecium*	Human stool	UK	D1	34
55	58155 (pat. 9)	*E. faecium*	Human urine	UK	D1	34
56	62899 (pat. 11)	*E. faecium*	Human urine	UK	D2	34
57	68521 (pat. 15)	*E. faecium*	Human urine	UK	D1	34
58	72801 (pat. 12)	*E. faecium*	Human wound	UK	D1	34
59	80103 (BC20)	*E. faecium*	Human blood	UK	D1	15
60	89407 (U22)	*E. faecium*	Human urine	UK	D1	15
61	26712 (pat. 1)	*E. faecium*	Human urine	UK	D1	34
62	38658 (pat. 2)	*E. faecium*	Human blood	UK	D1	34
63	42757 (pat. 3)	*E. faecium*	Human urine	UK	D1	34

Continued on following page

***Table 1**-Continued*

Strain No.	Strain	Enterococcal species	Source	Country	*Tn1546* -types	Reference
64	43088 (pat. 4)	*E. faecium*	Human urine	UK	D1	34
65	68140 (pat. 10)	*E. faecium*	Human urine	UK	D1	34
66	66925 (pat. 13)	*E. faecium*	Human urine	UK	D1	34
67	74198 (pat. 14)	*E. faecium*	Human pus	UK	D4	34
68	70040 (pat. 16)	*E. faecium*	Human urine	UK	D4	34
69	75436 (pat. 18)	*E. faecium*	Human pus	UK	D1	34
70	S1 (C2)	*E. faecium*	Sewage inlet A	UK	E1 *b*	15
71	S5 (L#3)	*E. faecium*	Sewage inlet B	UK	D3	15
72	S10 (C1)	*E. faecium*	Sewage inlet A	UK	E7	15
73	S17 (M7)	*E. faecium*	Sewage inlet B	UK	E3	15
74	S25 (M2)	*E. faecium*	Sewage inlet C	UK	G *b*	15
75	S26 (M3)	*E. faecium*	Sewage inlet C	UK	A2	15
76	S27 (Mix. 0.1#1)	*E. faecium*	Sewage inlet A	UK	A1	15
77	A1 (VF1)	*E. faecium*	Pig	UK	A2	15
78	A6 (Pig 22)	*E. faecium*	Pig	UK	A2	15
79	A10 (Pig 2,19)	*E. faecium*	Pig	UK	A2 *b*	15
80	C2 (Sim Chick)	*E. faecium*	Uncooked chick.	UK	B3	15
81	C3 (T2)	*E. faecium*	Uncooked chick.	UK	A1	15
82	C4 (Chicken 1)	*E. faecium*	Uncooked chick.	UK	B1	15
83	C5 (Grade A)	*E. faecium*	Uncooked chick.	UK	E4	15
84	C12 (VF4)	*E. faecium*	Turkey	UK	A1	15
85	C13 (VF7 alfa)	*E. faecium*	Duck	UK	A1	15
86	C14 (VF8)	*E. faecium*	Chicken	UK	A1	15
87	C15 (VF9)	*E. faecium*	Pony	UK	A1	15
88	VS1	*E. faecium*	Human	USA	F2 *b*	45
89	VS2	*E. faecium*	Human	USA	F2	45
90	VS3	*E. faecium*	Human	USA	F2	45
91	VS4	*E. faecium*	Human	USA	F1	45
92	VS5	*E. faecium*	Human	USA	F2	45
93	VS6	*E. faecium*	Human	USA	F2	45
94	VS7	*E. faecium*	Human	USA	F2	45
95	VS8	*E. faecium*	Human	USA	F2	45
96	VS9	*E. faecium*	Human	USA	F2	45
97	VS10	*E. faecium*	Human	USA	F2	45

pat. patient
a NL, The Netherlands; UK, United Kingdom; USA, United States.
b *Tn1546* types which were sequenced entirely.

respectively; see Table 2 and Fig. 1). Labeling of the PCR fragments and detection of hybrids were performed as described in the instructions for the ECL direct nucleic acid labeling and detection kit (Nycomed Amersham plc.).

DNA sequence analysis

The PCR products described below were purified with a Qiagen PCR purification kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. Subsequently, the purified PCR products were sequenced directly with the ABI PRISM Big Dye cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) on an ABI PRISM 377 DNA Sequencer (Perkin Elmer). All VRE isolates were analyzed for the point mutation in the *orf1*, *vanS*, and *vanX* genes. To determine the DNA sequence of the left end of the truncated

VanA transposon derivatives, type A2, B3, C, D1 to D4, E1 to E7, F1, F2, and G DNA fragments were amplified with T*n1546* primer 184.R, 1009.R, 1292.R, or 4511R in combination with I*S1216* primer IS1216V.B.

Table 2: *PCR and sequence primers used in this study*

Primer *a*	Sequence	Positions *b*
T*n1546* primers		
22.F	5'-GGATTTACAACGCTAAGCC	22–40
184.R	5'-ACCATATGTCGCCCTTAG	184–167
934.F	5'-TGTGGATTTGCATCTGC	934–950
1009.R	5'-ACGGTACAACATCTTCGTC	1009–991
1292.R	5'-TTACTCATGGATGTGGCC	1292–1275
1723.F	5'-ACAGGTGAGTCATCAGGC	1723–1740
1890.F	5'-TAAATAATCATAGTCGGCAGG	1890–1910
1913.R	5'-CGTCCTGCCGACTATG	1913–1898
1924.R	5'-TAGGAACTTGCACGTCCT	1924–1908
2768.F	5'-AGGATGGACTAACACCAATC	2768–2787
2880.R	5'-TGCTGTTCAATTAGCTGTTC	2880–2861
3514.F	5'-ACTGTAATGGCTGGTGTTAAC	3514–3534
3560.R	5'-TATCCGAATAAGATCTCGCT	3560–3542
3940.R	5'-ATTTATCAGATTATAGGGCCG	3940–3920
3992.F	5'-TTATTGTGGATGATGAACATG	3992–4012
4426.F	5'-AACGAGAAGCAGTTATCCC	4426–4444
4511.R	5'-TCGGAGCTAACCACATTC	4511–4494
5235.F	5'-ATATCACGTTGGACAAAGC	5235–5253
5374.R	5'-TTCATCGGTCATCTGCAC	5374–5357
5747.F	5'-ACGTTTAGGGTAGAGCTTCC	5747–5766
6039.F	5'-GTTTATGGATGTGAGCAGG	6039–6057
6113.R	5'-TATCGTTGCCATAACGC	6113–6097
6964.F	5'-AAAGGAGACAGGAGCATG	6964–6981
7035.R	5'-TTACGTCATGCTCCTCTGAG	7035–7017
7486.R	5'-CAAAAACAGGATAGGTAAACG	7486–7466
7875.F	5'-CCGCATTGTACTGAACG	7875–7891
7986.R	5'-CAAGCGGTCAATCAGTTC	7986–7969
8544.F	5'-GCATATAGCCTCGAATGG	8544–8561
8691.R	5'-TTACATACGTCGGGTTTCC	8691–8673
8969.R	5'-GATTGTGCCGTTTTGC	8969–8954
9519.F	5'-ACCAGCAGGTTATAGTGAGC	9519–9538
9580.R	5'-TCGTCAAGCTTGATCCTAC	9580–9562
9970.R	5'-GCCATCCTTACCTCCTTG	9970–9953
10716.R	5'-TTTTCCCCTCACTTCACAC	10716–10698
10778.F	5'-TTTAGTGCTGAGGAATTGG	10778–19796
I*S1216V* primers		
IS1216V.A	5'-GGAAAGCAATTTCAGCAG	254–271
IS1216V.B	5'-TCGATGCAGATGGTTTAAC	516–534
IS1216V.C	5'-CACTTGTAATAGAGGGGGC	659–641
IS1216V.D	5'-TGGGATTCCCAATAATACC	895–913
IS1216V.E	5'-AGCTTAAATCATAGATACCGTAAGG	913–935
IS1216V.F	5'-TTCATCGTCATTCCTCCTCCTG	243–225

a The names of the T*n1546* primers indicate the position of the first nucleotide and the orientation of the primer (F, forward; R, reverse). *b* The positions of the T*n1546* primers are based on the sequence of T*n1546* (GenBank and EMBL accession no. M97297). The positions of the I*S1216V*primers are based on the sequence of I*S1216V* (GenBank and EMBL accession no. L40841).

The exact integration site and orientation of I*S1216V* in the *vanX-vanY* intergenic region were determined by amplifying a DNA fragment with primers 7875.F and 10716.R, and the sequence was determined with the I*S1216V* primers IS1216.E and IS1216.F. Finally, all VRE isolates carrying T*n1546* types F1 and F2 were

analyzed for the mutation in the *vanA* and *vanY* genes, as determined with isolate VS1, by sequencing the corresponding region.

Results

RFLP analysis of Tn1546-like elements

In order to identify polymorphic regions in the vancomycin-resistant transposon T*n1546*, 97 different *vanA* gene-carrying VRE (Table 1) isolated from different sources were analyzed by means of RFLP analysis.

Seven different RFLP patterns, types A to G, were detected (Fig. 1). The banding pattern of type A was identical to the predicted pattern for the published sequence of T*n1546* [5]. For types B, D, E, and G, an additional fragment of approximately 1,800 bp was present, suggesting an insertion. The lack of fragment 1 or 6 in types C to G suggests that these transposons had deletions from the left end. Furthermore, the lack of fragment 2 in types D and G suggests polymorphism at the right end of the transposon. No polymorphism was found among the restriction fragments from the central regions of T*n1546*, *van*R, *van*S, *van*H, and *van*A. The high molecular-mass bands present in types A to E and G represent DNA fragments flanking the VanA transposon. The absence of flanking fragments in type F is partially explained by deletions from the left end of the transposon (see above). In addition, the flanking fragment at the right end appeared to migrate at the position of fragment 4, while the original fragment 4 in lane F was absent, probably due to a rearrangement in this region.

Sequence analysis of the VanA transposons of representatives of the seven RFLP types

Thirteen representatives of the seven different T*n1546* RFLP types (strains 3, 17, 22, 23, 24, 26, 42, 48, 51, 70, 74, 79, and 88 [Table 1]) were analyzed in more detail by determining the nucleotide sequence of the entire transposon. Overlapping internal fragments of T*n1546* were amplified and were subsequently sequenced by using combinations of 35 T*n1546*-specific primers (Table 2). The sequences that were obtained were compared with the published sequence of T*n1546*. Consistent with the RFLP analysis, RFLP types C, D, E, F, and G lacked sequences at the left end of the transposon. In order to determine the exact left ends of the truncated T*n1546*-related elements, DNA fragments were amplified

with a combination of T*n1546*-derived primers and primers based on the insertion element I*S1216*V.I*S1216V* was found to be located upstream from T*n1546* in strains of RFLP types D, E, F, and G. In strains of RFLP type C, no I*S1216V* was present upstream of the transposon, so that the exact left end of the transposon could not be determined and was estimated from the RFLP data to be between 1,275 and 2,842 bp.

The major rearrangements among the 13 strains investigated were the insertion of a I*S1216*V-I*S3*-like element at the left end of the transposon (types A2 and B3), the insertion of one or two copies of I*S1216V* (types B and D to G), the insertion of one copy of I*S1251* (type F), deletions associated with IS insertions downstream of *vanX* (types D to G), and at the left end of the transposon, deletions that affect the transposase or the resolvase gene (types C to G) (Fig. 2).

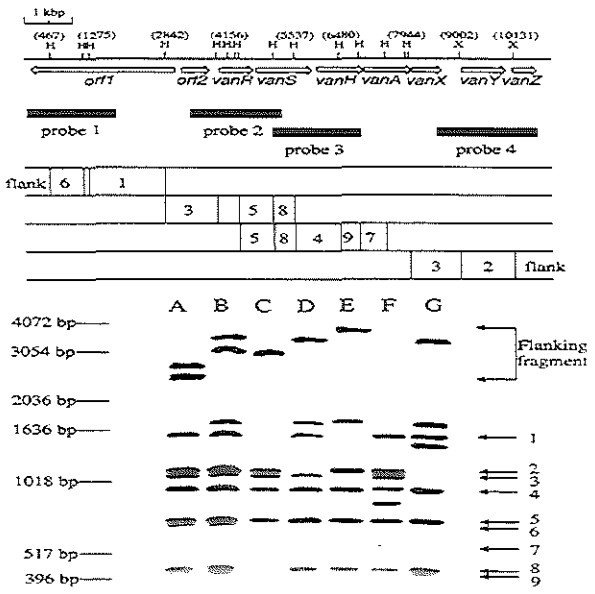


Figure 1: *RFLP analysis and physical and genetic maps of Tn1546. The position and direction of transcription of genes and open reading frames (orf's) are indicated with open arrows. Black horizontal bars indicate the position of internal Tn1546 fragments used as probes (probes 1 to 4). The numbers 1 to 9 represent the restriction fragments visualized after hybridization with the Tn1546-specific probes 1 to 4 and are indicated on the right side of the blot. The positions of the molecular size markers are indicated on the left side of the blot. Letters above the lanes represent the Tn1546 RFLP types. Only the restriction enzyme recognition sites relevant for this study are shown. H, HaeIII; X, XbaI. The positions of some restriction sites are indicated in parentheses.*

Insertion of the I*S1216*V-I*S3*-like element at the left end of the transposon and insertion of I*S1216V* in the *vanXY* intergenic region have been described previously [36, 49]. It is interesting that copies of I*S1216V* inserted in the *vanXY* intergenic region in strains 24, 42, 48, 70, and 74, which were completely sequenced, contained a synonymous T-to-C point mutation at position 826

relative to the published sequence of *IS1216V* (GenBank accession no. L40841). In all strains with *IS1216V* insertions except strains in which the IS insertions were accompanied by small adjacent deletions, an 8-bp duplication of the target sequence (CCCATTGT) was found. Insertion of *IS1216V* in the *vanXY* intergenic region also explained the presence of the additional 1.8-kbp fragment in types B, D, E, and G (Fig. 1). Insertion of *IS1251* in the *vanSH* intergenic region resulted in an 8-bp duplication of the target sequence, ATAATTTT. Furthermore, insertion of *IS1251* in this region explained the absence of fragment 4 in lane F (Fig. 1). Insertion of *IS1251* at this site has also been described previously (27). Furthermore, DNA polymorphism due to point mutations in *orf1* (1226), *vanS* (4847), *vanA* (7658), *vanX* (8234), and *vanY* (9692) were found (Fig. 2). Altogether 11 different *Tn1546* types were distinguished among the 13 strains whose transposons were sequenced: type A1 (which is *Tn1546*), A2, A3, A4, B1, C, D1, E1, E2, F2, and G (Fig. 2).

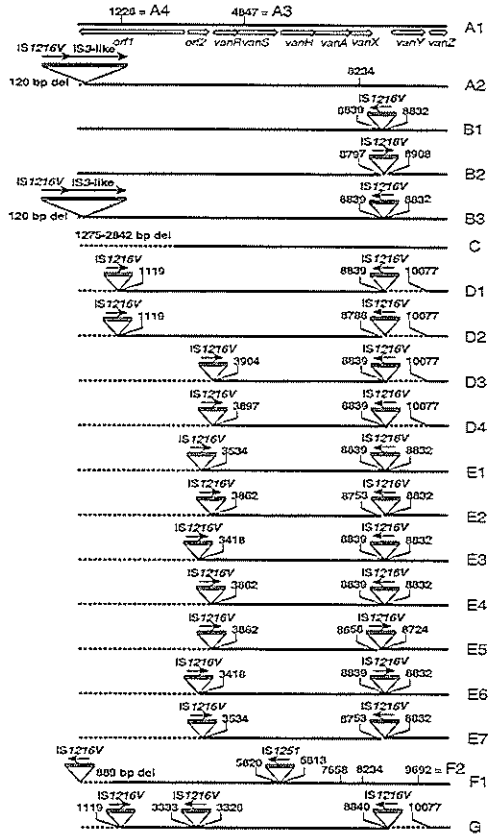


Figure 2: *Genetic maps of 22 Tn1546 types. The thick horizontal lines represent the Tn1546 types A1 to A4, B1 to B3, C, D1 to D4, E1 to E7, F1, F2, and G. The positions of genes and open reading frames (orf's) and the direction of transcription are depicted with open arrows. Dotted boxes represent IS elements. The positions of the first nucleotide upstream and the first nucleotide down-stream from the IS insertion sites are depicted. Filled arrows indicate the transcriptional orientations of the inserted IS elements. Deletions (del) are indicated by dotted lines. The positions of base pair mutations are indicated above the different Tn1546 types: 1226, T→A (K→stop); 4847, T→C; 7658, T→C (V→A); 8234, G→T (K→N); 9692, C→T (P→L).*

Analysis of the polymorphic regions in Tn1546 in other isolates of VRE.

We analyzed the polymorphic regions of T*n1546* of 87 additional VRE which were initially examined by RFLP analysis. The presence of the point mutations in the *van*X, *van*S, and *orf1* genes, the exact integration sites and the orientations of I*S1216V* and I*S1251*, the deletions surrounding the I*S1216V* insertion site, and the size of the left-end deletion were assessed by means of DNA sequencing. Furthermore, isolates of VRE carrying the type F transposon were analyzed for the point mutation in the *vanA* and the *vanY* genes. DNA sequencing finally distinguished 22 different transposon types. RFLP type A could be subdivided into four subtypes (subtypes A1 to A4), type B could be subdivided into three subtypes (subtypes B1 to B3), type D could be subdivided into four subtypes (subtypes D1 to D4), type E could be subdivided into seven subtypes (subtypes E1 to E7), and type F could be subdivided into two subtypes (subtypes F1 and F2). Types C and G could not be subdivided. On the basis of RFLP analysis, types D3 and D4 were initially designated E subtypes since they both lacked fragments 6, 1, and 3 at the left ends of their transposons. However, since these two types also lacked the *vanY* gene, which is indicative of type D, they were renamed D3 and D4. The identification of I*S1216V* in the *vanity* intergenic region in types D1, D2, and D4 in strains 46 to 69 contradicts the results published previously by Jensen et al. [39] since in that study the same strains were partly analyzed, but no sequence or size variation was observed in the amplicons of the *vanity* intergenic region.

Glycopeptide susceptibility patterns of isolates

The MICs of vancomycin, teicoplanin, and avoparcin for the 97 different isolates were determined by the agar dilution method. Generally, no association was found between the resistant phenotype and the transposon genotype. All isolates were resistant to vancomycin (MICs at which 50% [MIC50] and 90% [MIC90] of isolates are inhibited, 512 and 1,024 mg/ml, respectively) and avoparcin (MIC50 and MIC90, 256 and 1,024 mg/ml, respectively). Exceptions were strains with deletions of the *vanY* gene (types D1, D2, D3, D4, and G). These strains were less resistant to teicoplanin (MIC50 and MIC90, 16 and 64 mg/ml, respectively) than strains belonging to the other types (MIC50 and MIC90, 128 and 256 mg/ml, respectively). It is conceivable that the deletion of

vanY affects the transcription of *vanZ*, resulting in a lower MIC of teicoplanin, because *vanZ* has been shown to be involved in teicoplanin resistance [10, 50].

Table 3: *Ribotypes, PFGE types, and Tn1546 types of VRE isolated from the John Radcliffe Hospital, Oxford, and the Cook County Hospital, Chicago*

Strain No.	*Source*	*City*	*Ribotype*	*PFGE Type*	*Tn1546 Type*
48	Human stool	Oxford	2	G′	D1
49	Human stool	Oxford	4	U	D1
50	Human stool	Oxford	11	Q	D1
53	Human stool	Oxford	1	H′	D1
54	Human stool	Oxford	1	H	D1
55	Human urine	Oxford	1	I	D1
56	Human urine	Oxford	4	G	D2
57	Human urine	Oxford	5	P	D1
58	Human wound	Oxford	2	F	D1
59	Human blood	Oxford	6	P′	D1
60	Human urine	Oxford	6	H	D1
61	Human urine	Oxford	1	H	D1
62	Human blood	Oxford	1	H	D1
63	Human urine	Oxford	1	H	D1
64	Human urine	Oxford	1	H	D1
65	Human urine	Oxford	1	H	D1
66	Human urine	Oxford	1	H	D1
67	Human pus	Oxford	1	H″	D4
68	Human urine	Oxford	1	H	D4
69	Human pus	Oxford	1	H	D1
51	Human stool	Oxford	8	A	C
52	Human stool	Oxford	9	R	A1
88	Human *c*	Chicago	ND *d*	UU	F2
89	Human *c*	Chicago	ND	VV	F2
90	Human *c*	Chicago	ND	WW	F2
91	Human *c*	Chicago	ND	XX	F1
92	Human *c*	Chicago	ND	YY	F2
93	Human *c*	Chicago	ND	ZZ	F2
94	Human *c*	Chicago	ND	AAA	F2
95	Human *c*	Chicago	ND	BBB	F2
96	Human *c*	Chicago	ND	CCC	F2
97	Human *c*	Chicago	ND	DDD	F2

a Ribotypes have been described previously [34].
b Interpretation of banding patterns is according to Tenover et al. [47].
c The strains were from multiple body sites.
d ND, not determined.

Tn1546 types among VRE isolated from hospitalized patients

Our collection of VRE comprised two sets of strains isolated from hospitalized patients. One set of 22 VRE originated from an outbreak at the John Radcliffe Hospital in Oxford, United Kingdom [34]. These 22 isolates represented eight different ribotypes and 13 different PFGE types (Table 3), which suggests that at least 13 different enterococcal strains were involved in this outbreak. In contrast, 17 of the 22 isolates contained the same D1 type of T*n1546* (Table 3). Furthermore, an additional three strains harbored either T*n1546* type D2 or T*n1546* type

D4, which could be derived from D1 by a single DNA rearrangement (Fig. 3). T*n1546* type D1 was found among nine different strain types. A second set of 10 strains originated from a 7-week survey for VRE contamination at Cook County Hospital, Chicago, Ill. [45, 46]. All 10 *E. faecium* strains had different PFGE types (Table 3). Interestingly, all isolates except one contained the same T*n1546* derivative, T*n1546* type F2. One isolate, isolate VS4, contained the type F1 transposon, which differed from type F2 by a single base pair. The data on the prevalence of transposon types in the Oxford and Chicago hospitals suggest the possibility of horizontal transmission of vancomycin resistance transposon types D1 and F, respectively, among different enterococcal hosts.

Table 4: *Distribution of 22 different Tn1546 derivatives among 97 isolates of VRE from human and animal sources*

	No. of isolates from the following sources *a* :					
T*n1546* Type	Human (NL) (n=21)	Animal (NL) (n=24)	Human (UK) (n=24)	Animal (UK) (n=18)	Human (USA) n=10)	Total
A1	7	5	2	6		20
A2	13	12		4		29
A3		1				1
A4		1				1
B1		1		1		2
B2		1				1
B3				1		1
C			1			1
D1			17			17
D2			1			1
D3				1		1
D4			2			2
E1				1		1
E2		1	1			2
E3		1		1		2
E4				1		1
E5		1				1
E6	1					1
E7				1		1
F1					1	1
F2					9	9
G				1		1
Total	21	24	24	18	10	97

a NL, The Netherlands; UK, United Kingdom; USA, United States; n, total number of isolates from that source

Discussion

To facilitate understanding of the molecular epidemiology of vancomycin resistance, we undertook a detailed study of the molecular diversity and the evolutionary relationships of T*n1546*-like elements in enterococci from humans and animals. Knowledge of the diversity of T*n1546* is important for distinguishing between the dissemination of a single VRE clone and the transmission of a particular T*n1546* type through a genetically divergent population of enterococci. Typing of VRE by methods such as PFGE and ribotyping has shown the clonal dissemination of VRE in hospitals [30, 32, 51, 52]. However, transmission of particular T*n1546* types has not been documented before. Nevertheless, various studies suggest that this occur since genetic divergence in VRE genomes was found among strains isolated from epidemics caused by VRE [27-31].

In this study we have identified and characterized polymorphic regions in T*n1546*-like elements from 97 VRE originating from animal and human sources. By means of a combination of RFLP analysis and DNA sequencing, 22 different T*n1546*-like elements were distinguished. Three types of polymorphisms were found: point mutations, insertions of IS elements, and deletions generally associated with the insertion of IS elements. The point mutations were located in the *orf*1, *van*S, *van*A, *van*X, and *vanY* genes. The only point mutation described previously is in the *vanX* gene at position 8234 [10, 39]. Jensen et al. [39] also found this mutation in the *vanX* gene in three strains that we have also analyzed, strains 77 to 79.

The vast majority (74 of 97) of strains contained one to three copies of the insertion sequence I*S1216V* inserted in the vancomycin resistance transposon. Insertion of this IS element in the *vanXY* intergenic region and its presence on either side of T*n1546* have been described previously [36, 49, 53]. The presence of IS element insertions was often associated with deletions, a phenomenon which has been described previously [54, 55]. Thirty isolates containing the type A2 and B3 VanA transposons had similar genetic organizations at the left end of the VanA transposon, as in strain GUC described by Handwerger and Skoble [49]. In these types as well as in strain GUC, a copy of an I*S1216*V-I*S3* like element is present at the left end of the VanA transposon, resulting in a deletion of the first 120 bp. In strain GUC the T*n1546*-like element is located on a large chromosomal mobile element designated T*n5482*. Preliminary analysis of two

representative isolates carrying type A2 transposons indicated a chromosomal location of the VanA element (data not shown), which is similar to the case for strain GUC, which may suggest that type A2 and B3 VanA transposons are part of a larger chromosomal mobile element. In strains 77 to 79 the presence of the IS*1216*V-IS*3* element at the left end of the T*n1546*-like element is consistent with the finding of Jensen et al. [39]. In addition to IS*1216*V, insertions of IS*1251* in the *vanSH* intergenic region were found. Although the insertion of IS*1251* at this site was published previously, the transposon in *E. faecium* GUC described by Handwerger and colleagues [38, 49] was clearly distinct from the T*n1546* type F transposon, since no insertion of an IS*1216*V-IS*3* like element was present directly upstream from T*n1546* in the type F transposons.

Remarkable was the finding that 72 (74%) of the analyzed strains (types A2, B3, C, D1 to D4, E1 to E7, F1, F2, and G) carried small or large deletions in the transposase and resolvase regions of the T*n1546*-like transposon. A similar finding has recently been reported by others [43]. Although it is expected that deletions in the transposase and resolvase regions which abolish transposition may affect the dissemination of truncated T*n1546*-like elements, other studies have shown that T*n1546*-like elements are often part of chromosomal mobile elements [49] or plasmids that can be mobilized (28).

In this study we investigated in detail the polymorphism in T*n1546* with the aim of exploiting differences in this genetic element for future studies on the epidemiology of vancomycin resistance. Because we examined a large number of strains from a variety of sources, some preliminary conclusions may be drawn. T*n1546* types A1 and A2 were the most prevalent in The Netherlands both among isolates from humans and among isolates from farm animals (Table 4), suggesting an epidemiological link between animal and human reservoirs. The presence of identical VanA transposons in VRE isolated from humans and animals has also been described recently in Denmark and the United Kingdom [39, 43]. In VRE from hospitalized patients in the United States we found transposons which contain insertions of IS*1251*. So far this IS element was been found only by Handwerger et al. [39], Jensen et al., and MacKinnon et al. [40] in isolates from U.S. patients.

It is intriguing that the majority of the transposon types found in hospitals in the United Kingdom and the United States (types D1 and F2) have no counterpart in animals. For the U.S. isolates, this is explained by the fact that so

far no VRE have been isolated from animals in the United States. The fact that no D types were found among the isolates from animals in the United Kingdom may suggest that once it was introduced in the Oxford hospital the VanA transposon has evolved independently from the transposons from counterpart strains from animals. This is consistent with the scheme presented in Fig. 3. Figure 3 depicts a hypothetical evolutionary scheme in an attempt to explain the relationships between the 22 transposon types. In Fig. 3 transposon types D (types D1, D2, and D4) and F (types F1 and F2) are located separately from the majority of the subtypes found outside hospitals. In the scheme presented in Fig. 3 we assume that the various T*n1546* variants evolved by base pair substitutions, transpositions, and deletions. We did not include homologous recombination events, although they could lead to a more parsimonious phylogeny. The preliminary data on region specificity suggest that geographic isolation contributed to differences in the prevalence of particular T*n1546* subtypes at different geographic sites.

The combination of the polymorphism in T*n1546* and the epidemiological data indicate that the DNA polymorphism among T*n1546* variants can be exploited successfully for the tracing of the routes of transmission of vancomycin resistance genes. Indicative of this is the finding of identical or closely related VanA transposon types among genetically different enterococci in the Oxford hospital as well as in the hospital in Chicago. Studies are in progress to use the tools developed in this study to investigate in detail the prevalence of subtypes of T*n1546* among humans and animals. This may resolve the controversial issue of the spillover of vancomycin resistance to humans from the animal reservoir due to the use in animal husbandry of glycopeptide antibiotics, such as avoparcin, for growth promotion. Avoparcin has been used in Europe for more than 20 years, but it is anticipated that the current ban on the veterinary use of this antibiotic will also lead to an overall decrease in the frequency of vancomycin resistance among enterococci colonizing the human digestive tract.

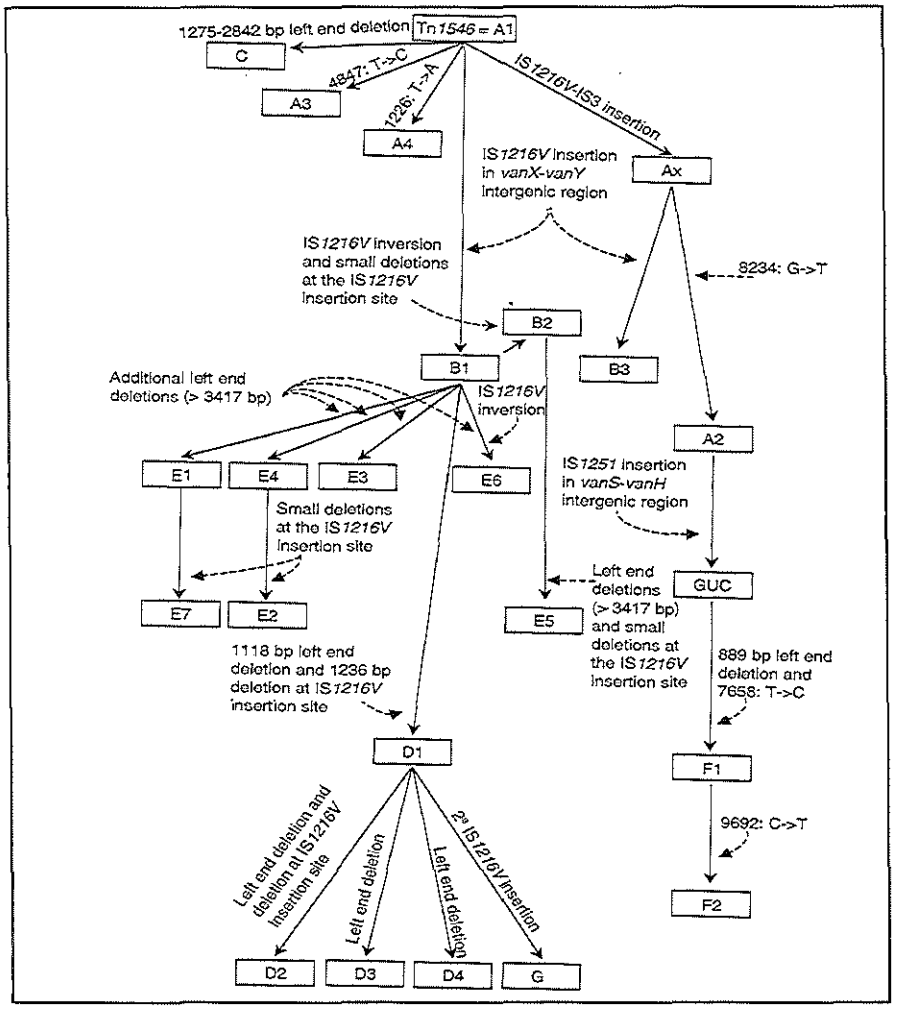


Figure 3: *Hypothetical evolutionary scheme for the various Tn1546 derivatives characterized in this study from the archetypal transposon Tn1546 (type A1) as described by Arthur et al. in 1993 (5). Boxes represent the different Tn1546 types. Filled arrows indicate the transition of Tn1546 type A1 to the other Tn1546 types. The different DNA rearrangements, insertions, deletions, and point mutations are indicated. Strain GUC has been described by Handwerger et al. (27)*

Acknowledgments

We are grateful to Marc Bonten, Cook County Hospital, Chicago, Ill., for providing the strains from the United States; Hubert Endtz, Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands, for providing the Dutch clinical isolates; J. Zoe Jordens, John Radcliffe Hospital, Oxford, United Kingdom, for providing the English strains; and Ellen Stobberingh and Ton van den Bogaard, University Hospital Maastricht, Maastricht, The Netherlands, for providing the isolates from Dutch pigs. We thank Remco van den Hoek and Ans van Veen from the National Institute of Public Health, Bilthoven, The Netherlands, and Kees Veldman from the DLO-Institute for Animal Science and Health, Lelystad, The Netherlands, for technical support. Finally, we thank Han de Neeling and Tjeerd Kimman from the National Institute of Public Health, Bilthoven, The Netherlands, for helpful discussions and critical reading of the manuscript.

References

1. Centers for Disease Control and Prevention. 1993. Nosocomial enterococci resistant to vancomycin—United States, 1989–1993. Morbid. Mortal. Weekly Rep. 42:597–599.
2. McDonald, L. C., M. J. Kuehnert, F. C. Tenover, and W. R. Jarvis. 1997. Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. Emerg. Infect. Dis. 3:311–317.
3. Coque, T. M., J. F. Tomayko, S. C. Ricke, P. C. Okhyusen, and B. E. Murray. 1996. Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob. Agents Chemother. 40: 2605–2609.
4. Endtz, H., N. van den Braak, A. van Belkum, J. A. J. W. Kluytmans, J. G. M. Koeleman, L. Spanjaard, A. Voss, A. J. L. Weersink, C. M. J. E. Vanden broucke-Grauls, A. G. M. Buiting, A. van Duin, and H. A. Verbrugh. 1997. Fecal carriage of vancomycin-resistant enterococci in hospitalized patients and those living in the community in The Netherlands. J. Clin. Microbiol. 35: 3026–3031.
5. Gordts, B., H. Van Landuyt, M. Ieven, P. Vandamme, and H. Goossens. 1995. Vancomycin-resistant enterococci colonizing the intestinal tracts of hospitalized patients. J. Clin. Microbiol. 33:2842–2846.
6. Suppola, J. P., L. Volin, V. V. Valtonen, and M. Vaara. 1996. Overgrowth of *Enterococcus faecium* in the feces of patients with hematologic malignancies. Clin. Infect. Dis. 23:694–697.
7. Schouten, M. A., J. A. A. Hoogkamp-Korstanje, and A. Voss. 1997. Controlling glycopeptide-resistant enterococci. Clin. Microbiol. Infect. 3:592–593.
8. Torres, C., J. A. Reguera, M. J. Sanmartin, J. C. Perez-Diaz, and F. Baquero. 1994. *van*A-mediated vancomycin-resistant *Enterococcus* spp. in sewage. J. Antimicrob. Chemother. 33:553–561.
9. Van Belkum, A., N. van den Braak, R. Thomassen, H. Verbrugh, and H. Endtz. 1996. Vancomycin-resistant enterococci in cats and dogs. Lancet 348: 1038–1039.
10. Van den Bogaard, A. E., L. B. Jensen, and E. E. Stobberingh. 1997. Vanco-mycinresistant enterococci in turkeys and farmers. N. Engl. J. Med. 337: 1558–1559.
11. Van den Bogaard, A. E., P. Mertens, N. H. London, and E. E. Stobberingh. 1997. High prevalence of colonization with vancomycin- and pristinamycin-resistant enterococci in healthy humans and pigs in The

Netherlands: is the addition of antibiotics to animal feeds to blame? J. Antimicrob. Chemother. 40:454–456.

12. Van der Auwera, P., N. Pensart, V. Korten, B. E. Murray, and R. Leclercq. 1996. Influence of oral glycopeptides on the fecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J. Infect. Dis. 173: 1129–1136.
13. Aarestrup, F. M., P. Ahrens, M. Madsen, L. V. Pallesen, R. L. Poulsen, and H. Westh. 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.
14. Bager, F., M. Madsen, J. Christensen, and F. M. Aarestrup. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev. Vet. Med. 31:95–112.
15. Bates, J., J. Z. Jordens, and D. T. Griffiths. 1994. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J. Antimicrob. Chemother. 34:507–514.
16. Chadwick, P. R., N. Woodford, E. B. Kaczmarski, S. Gray, R. A. Barrell, and B. A. Oppenheim. 1996. Glycopeptide-resistant enterococci isolated from uncooked meat. J. Antimicrob. Chemother. 38:908–909.
17. Devriese, L. A., M. Ieven, H. Goossens, P. Vandamme, B. Pot, J. Hommez, and F. Haesebrouck. 1996. Presence of vancomycin-resistant enterococci in farm and pet animals. Antimicrob. Agents Chemother. 40:2285–2287.
18. Klare, I., H. Heier, H. Claus, R. Reissbrodt, and W. Witte. 1995. vanA-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry. FEMS Microbiol. Lett. 125:165–171.
19. Van den Braak, N., A. van Belkum, M. van Keulen, J. Vliegenthart, H. A. Verbrugh, and H. P. Endtz. 1998. Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in The Netherlands. J. Clin. Microbiol. 36:1927–1932.
20. Wegener, H. C., M. madsen, N. Nielsen, and F. M. Aarestrup. 1997. Isolation of vancomycin resistant Enterococcus faecium from food. Int.J. Food Microbiol. 35:57-66.
21. Aarestrup, F. M. 1995. Occurrence of glycopeptide resistance among Enterococcus faecium isolated from conventional and ecological poultry farms. Microb. Drug Resist. 1:255–257.
22. Klare, I., H. Heier, H. Claus, G. Bohme, S. Marin, G. Seltmann, R. Haken-beck, V. Antanassova, and W. Witte. 1995. *Enterococcus faecium* strains with *van*A-mediated high level glycopeptide resistance isolated from animal food-stuffs and faecal samples of humans in the community. Microb. Drug Resist. 1:265–272.
23. Power, E. G., Y. H. Abdulla, H. G. Talsania, W. Spice, S. Aathithan, and G. L. French. 1995. *vanA* genes in vancomycin-resistant clinical isolates of *Oerskovia turbata* and *Arcanobacterium (Corynebacterium) haemolyticum*.J. Antimicrob. Chemother. 36:595–606.
24. Poyart, C., C. Pierre, G. Quesne, B. Pron, P. Berche, and P. Trieu-Cuot. 1997. Emergence of vancomycin resistance in the genus *Streptococcus*: char-acterization of a *vanB* transferable determinant in *Streptococcus bovis*. Antimicrob. Agents Chemother. 41:24–29.
25. Fontana, R., M. Ligozzi, C. Pedrotti, E. M. Padovani, and G. Cornaglia. 1997. Vancomycin-resistant *Bacillus circulans* carrying the *vanA* gene responsible for vancomycin resistance in enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 16:473–474.
26. Noble, W. C., Z. Virani, and R. G. Cree. 1992. Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Lett. 72:195–198.
27. Bingen, E. H., E. Denamur, N. Y. Lambert-Zechovsky, and J. Elion. 1991. Evidence for the genetic unrelatedness of nosocomial vancomycin-resistant *Enterococcus faecium* strains in a pediatric hospital. J. Clin. Microbiol. 29: 1888–1892.
28. Clark, N. C., R. C. Cooksey, B. C. Hill, J. M. Swenson, and F. C. Tenover. 1993. Characterization of glycopeptide-resistant enterococci from U.S. hospitals. Antimicrob. Agents Chemother. 37:2311–2317.

29. Hall, L. M., B. Duke, M. Guiney, and R. Williams. 1992. Typing of *Enterococcus* species by DNA restriction fragment analysis. J. Clin. Microbiol. 30: 915–919.
30. Mato, R., H. Delencastre, R. B. Roberts, and A. Tomasz. 1996. Multiplicity of genetic backgrounds among vancomycin-resistant *Enterococcus faecium* isolates recovered from an outbreak in a New York City Hospital. Microb. Drug Resist. 2:309–317.
31. Morris, J. G., Jr., D. K. Shay, J. N. Hebden, R. J. McCarter, Jr., B. E. Perdue, W. Jarvis, J. A. Johnson, T. C. Dowling, L. B. Polish, and R. S. Schwalbe. 1995. Enterococci resistant to multiple antimicrobial agents, including vancomycin. Establishment of endemicity in a university medical center. Ann. Intern. Med. 123:250–259.
32. Biavasco, F., A. Miele, C. Vignaroli, E. Manso, R. Lupidi, and P. E. Varaldo. 1996. Genotypic characterization of a nosocomial outbreak of VanA *Enterococcus faecalis*. Microb. Drug Resist. 2:231–237.
33. Eliopoulos, G. M., C. B. Wennersten, H. S. Gold, T. Schulin, M. Souli, M. G. Farris, S. Cerwinka, H. L. Nadler, M. Dowzicky, G. H. Talbot, and R. C. Moellering. 1998. Characterization of vancomycin-resistant *Enterococcus faecium* isolates from the United States and their susceptibility in vitro to dalfopristin-quinupristin. Antimicrob. Agents Chemother. 42:1088–1092.
34. Jordens, J. Z., J. Bates, and D. T. Griffiths. 1994. Faecal carriage and nosocomial spread of vancomycin-resistant *Enterococcus faecium*. J. Antimi-crob. Chemother. 34:515–528.
35. Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin. 1993. Characterization of T*n1546*,a T*n*3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. 175:117–127.
36. Haaheim, H., K. H. Dahl, G. S. Simonsen, O. Olsvik, and A. Sundsfjord. 1998. Long PCRs of transposons in the structural analysis of genes encoding acquired glycopeptide resistance in enterococci. BioTechniques 24:432–437.
37. Arthur, M., F. Depardieu, G. Gerbaud, M. Galimand, R. Leclercq, and P. Courvalin. 1997. The VanS sensor negatively controls VanR-mediated tran-scriptional activation of glycopeptide resistance genes of T*n1546* and related elements in the absence of induction. J. Bacteriol. 179:97–106.
38. Handwerger, S., J. Skoble, L. F. Discotto, and M. J. Pucci. 1995. Heterogeneity of the *vanA* gene cluster in clinical isolates of enterococci from the northeastern United States. Antimicrob. Agents Chemother. 39:362–368.
39. Jensen, L. B., P. Ahrens, L. Dons, R. N. Jones, A. M. Hammerum, and F. M. Aarestrup. 1998. Molecular analysis of T*n1546* in *Enterococcus faecium* isolated from animals and humans. J. Clin. Microbiol. 36:437–442.
40. MacKinnon, M. G., M. A. Drebot, and G. J. Tyrrell. 1997. Identification and characterization of I*S1476*, an insertion sequence-like element that disrupts VanY function in a vancomycin-resistant *Enterococcus faecium* strain. Anti-microb. Agents Chemother. 41:1805–1807.
41. Miele, A., M. Bandera, and B. P. Goldstein. 1995. Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in VanA isolates. Antimicrob. Agents Chemother. 39:1772–1778.
42. Werner, G., I. Klare, and W. Witte. 1997. Arrangement of the *vanA* gene cluster in enterococci of different ecological origin. FEMS Microbiol. Lett. 155:55–61.
43. Woodford, N., A.-M. A. Adebiyi, M.-F. I. Palepou, and B. D. Cookson. 1998. Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. Antimicrob. Agents Chemother. 42:502–508.
44. Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33:24–27. (Erratum, 33: 1434.)

45. Bonten, M. J. M., M. K. Hayden, C. Nathan, J. van Voorhis, M. Matushek, S. Slaughter, T. Rice, and R. A. Weinstein. 1996. Epidemiology of colonisation of patients and environment with vancomycin-resistant enterococci. Lancet 348:1615–1619.
46. Bonten, M. J. M., M. K. Hayden, C. Nathan, T. W. Rice, and R. A. Weinstein. 1998. Stability of vancomycin-resistant enterococcal genotypes isolated from long-term-colonized patients. J. Infect. Dis. 177:378–382.
47. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233–2239.
48. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and L. Struhl. 1989. Current protocols in molecular biology, section 2.4. Greene Publishing Associates, Brooklyn, N.Y.
49. Handwerger, S., and J. Skoble. 1995. Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 39:2446–2453.
50. Arthur, M., F. Depardieu, C. Molinas, P. Reynolds, and P. Courvalin. 1995. The *vanZ* gene of *Tn1546* from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. Gene 154:87–92.
51. Handwerger, S., B. Raucher, D. Altarac, J. Monka, S. Marchione, K. V. Singh, B. E. Murray, J. Wolff, and B. Walters. 1993. Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin, and gentamicin. Clin. Infect. Dis. 16:750–755.
52. Pegues, D. A., C. F. Pegues, P. L. Hibberd, D. S. Ford, and D. C. Hooper. 1997. Emergence and dissemination of a highly vancomycin-resistant *vanA* strain of *Enterococcus faecium* at a large teaching hospital. J. Clin. Microbiol. 35:1565–1570.
53. Heaton, M. P., L. F. Discotto, M. J. Pucci, and S. Handwerger. 1996. Mo-bilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. Gene 171:9–17.
54. Jilk, R. A., J. C. Makris, L. Borchardt, and W. S. Reznikoff. 1993. Implications of *Tn5*-associated adjacent deletions. J. Bacteriol. 175:1264–1271.
55. Wang, G., X. Xu, J. M. Chen, D. E. Berg, and C. M. Berg. 1994. Inversions and deletions generated by a mini-gamma delta (*Tn1000*) transposon. J. Bacteriol. 176:1332–1338.

CHAPTER 10

Host Specificity of Vancomycin-Resistant *Enterococcus faecium*

Rob J. L. Willems, Janetta Top, Nicole van den Braak[1], Alex van Belkum[1], Hubert Endtz[1], Dik Mevius[2], Ellen Stobberingh[3], Anthony van den Bogaard[3], and Jan D. A. van Embden.

National Institute of Public Health and the Environment, Bilthoven, The Netherlands
Erasmus University Medical Centre Rotterdam, The Netherlands[1]
ID-Lelystad Institute for Animal Science and Health, Lelystad, The Netherlands[2]
University Hospital Maastricht, The Netherlands[3]

The Journal of Infectious Diseases (2000) 182:816-823

Abstract

Amplified-fragment length polymorphism (AFLP) analysis was used to investigate the genetic relationships among 255 vancomycin-resistant *Enterococcus faecium* (VREF) strains isolated from hospitalized patients, non-hospitalized persons, and various animal sources. Four major AFLP genogroups (A–D) were discriminated. The strains of each taxon shared >65% of the restriction fragments. Most isolates recovered from non-hospitalized persons (75%) were grouped together with all pig isolates in genogroup A. Most isolates from hospitalized patients (84%), a subset of veal calf isolates (25%), and all isolates from cats and dogs clustered in genogroup C. Most isolates from chickens (97%) and turkeys (86%) were grouped in genogroup B, whereas most veal calf isolates (70%) clustered in genogroup D. Therefore, VREF strains are predominantly host-specific, and strains isolated from hospitalized patients are genetically different from the prevailing VREF strains present in the fecal flora of non-hospitalized persons.

Introduction

During the last 20 years, an increase in antimicrobial resistance among enterococci has been observed. In particular, vancomycin-resistant *Enterococcus faecium* (VREF) strains are often multidrug-resistant [1] and pose a serious threat in hospital infections, because infections with VREF strains are difficult to treat. In the United States, many hospitals reported a high prevalence of vancomycin resistance, but vancomycin-resistant enterococci seemed to be virtually absent in the community. This is in contrast to Europe, where VREF strains can easily be detected outside hospitals, in non-hospitalized persons and in farm animals [2–14]. The high prevalence of VREF strains in farm animals in Europe is thought to be the result of the use of the glycopeptide antibiotic avoparcin as an antimicrobial growth promoter [15]. Consequently, VREF strains from animal husbandry may enter the food chain and subsequently spread to humans. The most prevalent and best-studied vancomycin resistance transposon is T*n1546,* which confers high-level resistance to vancomycin and teicoplanin [16]. Detailed molecular analysis of T*n1546* isolated from different animal and human strains has shown considerable DNA polymorphism in T*n1546* and has revealed the presence of common T*n1546* types among animal- and human-derived VREF strains, irrespective of the host strain [9, 12, 14, 17–25]. In addition, VREF strains from pigs predominantly carried a particular T*n1546* type with a specific point mutation at position 8234, whereas enterococci isolated from poultry predominantly contained T*n1546* sub-types harboring an I*S1216V* insertion in the *vanX-vanY* intergenic region [9, 12, 14, 17, 18, 23]. Whether an animal reservoir of VREF actually poses a threat to humans depends on the ability of animal strains to colonize the human gut. In several studies, genetically indistinguishable enterococci have been found in animals and humans, suggesting that animal-derived enterococci may colonize the human gut [4, 9, 17, 22, 23, 26–28]. Recently, Berchieri [29] showed that ingestion of a VREF strain isolated from a chicken resulted in colonization of his own gut for 20 days. Both molecular and non-molecular typing schemes have been used to study the epidemiology of VREF [30–40]. Because of the high degree of strain differentiation, pulsed-field gel electrophoresis (PFGE) has been considered to be the reference standard for tracing the transmission of strains in hospital out-breaks [32–34]. However, by PFGE it is difficult to establish the degree of

genetic relatedness between epidemiologically nonrelated strains, because the banding patterns of such strains are often completely different. Therefore, it is unlikely that PFGE allows the disclosure of strain-characteristic differences in the host specificity of enterococci. Devriese and colleagues [41, 42] have shown that raffinose-positive *E. faecium* are typically associated with poultry, and sorbitol-positive *E. faecium* strains are associated with dogs, which suggests that certain enterococci are host-specific.

Amplified-fragment length polymorphism analysis (AFLP) is a novel technique that allows for the analysis of polymorphism among small restriction fragments [43]. An advantage of AFLP typing is that these small fragments originate from both variable and conserved DNA sequences, thus establishing a degree of genetic relatedness between strains that, by PFGE, would show no similarity at all. AFLP combines restriction enzyme analysis with polymerase chain reaction (PCR) and has been proven successful in studying the molecular epidemiology of various microorganisms [44–52]. In this study, we used AFLP to obtain insight in the genetic relationships among VREF strains isolated from infected patients, non-hospitalized persons, pets, and various farm animals.

Materials and Methods

Bacterial strains

Two hundred fifty-five *van*A-containing *E. faecium* isolates were analyzed. Eighty-seven were from hospitalized patients from 9 countries (United States: 38, two hospitals; United Kingdom: 24, two hospitals; The Netherlands: 11, two hospitals; France: 6, three hospitals; Israel: 3, two hospitals; Italy: 2, two hospitals; Czech Republic: 1; Germany: 1; Slovak Republic: 1) [7, 53–57], 24 were from non-hospitalized persons from 3 countries (United Kingdom: 3; Germany: 1; the Netherlands: 20) and 11 different cities [4, 9, 14], 12 were from pigs from 2 countries (United Kingdom: 3; The Netherlands: 9) and 12 different farms [4, 11, 58], 10 were from poultry farmers (10 different farms; provided by A. E. J. M. van den Bogaard, University of Maastricht, The Netherlands) and poultry slaughterers (The Netherlands, 1 processing plant, provided by A. E. J. M. van den Bogaard), 31 were from chickens from 2 countries (United Kingdom: 4; The Netherlands: 27) and 29 different farms (22

isolates were provided by A.E. J. M. van den Bogaard) [4, 12], 10 were from turkey farmers (the Netherlands: 10 different farms) and turkey slaughterers (the Netherlands: 1 processing plant) [9], 7 were from turkeys (The Netherlands: 7 different farms) [9], 9 were from veal calf farmers (the Netherlands: 4 different farms), 60 were from veal calves (The Netherlands: 56 different farms), 5 were from dogs, and 2 were from cats (The Netherlands) [28].

PFGE analysis of VREF strains

PFGE typing was done, as described elsewhere [12]. The DNA banding patterns were analyzed with BioNumerics, version 1.5 (Applied Maths, Kortrijk, Belgium). The Dice coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages was used for cluster analysis.

*Molecular characterization of T*n1546 *derivatives*

Characterization of the *vanA* gene–containing transposons was done by a combination of restriction fragment length polymorphism and DNA sequencing, as described elsewhere [14]. The T*n1546* types and subtypes—A1, A2, A3, B, C, D, E, and F—mentioned in this study have been described elsewhere [9, 14]. In short, type A1 is identical to the first-described VanA-containing transposon, T*n1546* [16]. Types A2 and A3 are characterized by the G→T point mutation at position 8234 and the T→C point mutation at position 4847, respectively. Type B transposons are characterized by an I*S1216V* insertion in the *van*X-*vanY* intergenic region, and type C is characterized by a left-end deletion. Types D and E combine the features of type B and C—that is, a left-end deletion and the I*S1216V* insertion. In addition, type D contains a deletion of the *vanY* gene. Some of the E subtypes contain a deletion of the *vanZ* gene. The F types are characterized by an I*S1251* insertion in the *van*S-*vanH* intergenic region and the G→T point mutation at position 8234. Furthermore, in some, but not all, F types, point mutations at the positions 7658 (T→C) and 9692 (C→T) are found. In this study, the B, D, E, and F types were not subdivided into the different subtypes that have been described elsewhere.

AFLP analysis of VREF strains

AFLP, as originally described by Vos et al. [43], is based on the ligation of 2 adapters to genomic restriction fragments, followed by a PCR-based amplification with adapter-specific primers. In this study, we used a single adapter instead of 2, resulting in self-ligation of the digested DNA, because the adapter will ligate to the cohesive ends generated by the 2 restriction enzymes. The main advantages of using a single adapter are less variation in peak intensities and improved reproducibility [44]. DNA was isolated, as described elsewhere [14], with the addition of a final ethanol precipitation step to further purify the DNA. The *Eco*RI-*Cfo*I adapter used in this study was prepared by mixing 2 oligonucleotide solutions (2 *mM* each), heating for 5 min at 957C, and allowing the mixture to cool for >10 min at room temperature. The structure of the *Eco*RI-*Cfo*I adapter was as follows: 5′ –AATTGTAAAACGACGGCCAGTAACG and CATTTTGCTGCC-GGTCATT-5′ (complementary sequence is underlined). For restriction ligation, a 5-mL mixture consisting of 23 One-Phor-All buffer (Amersham-Pharmacia Biotech, Uppsala, Sweden), 2 *mM* ATP, 5 U of *Eco*RI, 1 U of *Cfo*I, 1 U of T4 DNA ligase, and 0.8 *mM* adapter was prepared. After addition of 5 µL (10 ng) of genomic *E. faecium* DNA, the mixture was incubated for 2 h at 37° C, to allow simultaneous restriction and ligation. This resulted in the formation of circularized DNA molecules. For amplification, 90 µL of TE (20 *mM* Tris, 0.1 *mM* EDTA, pH 8.0) was added to the restriction ligation mixture, and, subsequently, 2 µL of this mixture was mixed with 0.25 µL (10 *m*M) of each AFLP primer (primer 1 [CfoI-G]: 5′ -CGACGGCCAGTAACGC-G; primer 2 [EcoRI-A]: 5′ -GGCCGTCGTTTTACAATTC-A) and 7.5 µL of AFLP amplification core mix (PE Biosystems, Foster City, CA). Primer 1 contained an extra selective base, G, and was labeled with the blue fluorescent dye 5-carboxyfluorescein. Primer 2 contained an extra selective base, A. PCR was done on a thermal cycler (model 9600; PE Biosystems). After the PCR mixture had been heated for 2 minutes 72° C, it was used for amplification by means of a "touchdown" PCR program as follows: 30 cycles of a 20-s denaturing step at 94° C, a 30-s annealing step (see below), and a 2-min extension step at 72° C, followed by incubation at 60 ° C for 30 min. The annealing temperature during the first cycle was 66° C and decreased 1° C at each cycle during the next 9 cycles. During the remaining 20 cycles, an annealing temperature of 56° C was used. The amplification products were separated on a 36-cm, 5% denaturing sequencing polyacrylamide gel (Long

Ranger Singel Pack; FMC Bioproducts, Rockland, ME) on a DNA sequencer (ABI PRISM 377; PE Biosystems). For this, 1 mL of reaction mixture was mixed with 1.25 µL of formamide, 0.5 µL of loading buffer (PE Biosystems), and 0.25 µL of the internal size marker (GeneScan-500 labeled with the red fluorescent dye 6-carboxy-x-rhodamine; PE Biosystems). The gel was run in 13 TBE (89 *mM* Tris, 89 *mM* boric acid, 2 *mM* EDTA, pH 8.2) for 3 h at 200 W (GeneScan run module 36D-2400).

Computer analysis of AFLP-generated patterns

The GeneScan collection software (PE Biosystems) was used to collect data during electrophoresis. After tracking and extraction of lanes, data were exported to BioNumerics (version 1.5; Applied Maths) for further analysis. Normalization was done by use of the reference positions of the internal DNA size marker GS-500. Fragments ranging in size from 50 to 500 nucleotides were used for comparison. The Pearson coefficient of similarity of AFLP curves was calculated with BioNumerics software (Applied Maths). Cluster analysis was done by the unweighted pair group method with arithmetic averages.

Results

Comparison of AFLP analysis with PFGE

In a pilot experiment, 25 VREF strains isolated from human patients, non-hospitalized persons, pigs, and chickens were subjected to AFLP analysis and PFGE. The number of AFLP bands with sizes of 50–500 bp was 13–37, with an average of 27 bands (figure 1). AFLP typing was found to be highly reproducible. The degree of similarity between quadruplicates was 95%–99% (data not shown). The strains clustered into 3 distinct AFLP groups (figure 1). Group A contained strains from non-hospitalized persons and pigs, group B from chickens, and group C from hospitalized patients. This apparent host-specific grouping was less distinct by PFGE typing (figure 1). Furthermore, the strains originating from a given host were more dissimilar by PFGE than by AFLP analysis, with the exception of strains HP3–HP12, which were recovered from a hospital outbreak [55]. These strains showed highly similar or identical PFGE patterns (similarity >82%) and have been considered to belong to a single clone [55]. As with PFGE typing, the strains from the hospital outbreak also showed identical AFLP

patterns (similarity >97%). In addition, 2 strains from non-hospitalized persons (NHP2 and NHP3) were indistinguishable by both PFGE and AFLP. The number of bands in PFGE patterns was 11–16, which is considerably less than in the AFLP patterns. Therefore, genomic changes in only a few restriction fragments may result in disproportionate differences in the PFGE banding patterns.

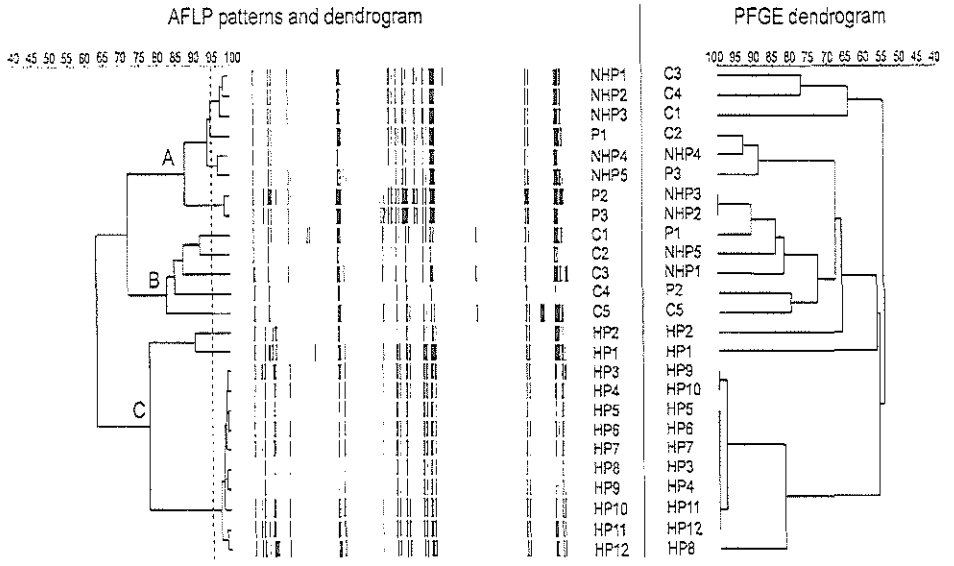


Figure 1: *Amplified-fragment length polymorphism (AFLP) patterns and dendrogram of 25 vancomycin-resistant Enterococcus faecium strains, typed by AFLP and pulsed-field gel electrophoresis (PFGE). Numbers on horizontal axes indicate % similarity, as determined by Pearson product-moment correlation coefficient and unweighted pair group method, with arithmetic averages for AFLP typing, and by Dice and unweighted pair group method, with arithmetic averages for PFGE typing. HP1–12, hospitalized patients; NHP1–5, non-hospitalized persons; P1–3, pigs; C1–5, chickens. A, B, and C in left dendrogram represent 3 genogroups. Dotted line depicts 95% similarity coefficient, above which strains were considered to be of identical AFLP type.*

Genogrouping and association with source of isolation

Two hundred fifty-five VREF strains recovered from different human and animal sources were subjected to AFLP typing. The strains from hospitalized patients were isolated from different sites, such as stool, blood, pus, urine, and ascites (table 1).

Table 1: *Isolation sites and genogrouping of vancomycin-resistant Enterococcus faecium strains recovered from hospitalized patients.*

	No. of strains isolated, by isolation site								
Genogroup	Ascites	Bile	Blood	Catheter	Pus	Skin	Urine	Stool	Unknown
A	—	—	—	—	—	1	1	2	—
B	2	—	—	—	1	—	1	5	1
C	3	3	10	2	3	1	27	24	—
Total	5	3	10	2	4	2	29	31	1

The result of the grouping by AFLP of these VREF strains is shown in figure 2. Four main groups (groups A–D) were discernible, and the strains within each group shared >65% of their restriction fragments. As described above, in the pilot experiment, grouping of the strains by AFLP was clearly associated with the source of the strains. Most of the 87 strains from hospitalized patients (84%) clustered in genogroup C (figures 2, *3*A). Within this genogroup, 2 subgroups, C1 and C2, were discerned, each containing strains with indistinguishable AFLP banding patterns (similarity >95%). Group C1 strains were isolated during a 32-month period at 6 Detroit metropolitan area hospitals [54], and group C2 strains were isolated during a 3-month period at the John Radcliffe Hospital in Oxford, United Kingdom [55]. Strains in subgroups C1 and C2 are most likely repetitive isolates of a single strain. The same is probably true for 2 sets of 2 strains in genogroup B and for 3 other sets of 2 strains in genogroup C, which have indistinguishable AFLP patterns (similarity >95%) and originated from the same hospital. When these repetitive isolates were counted only once, 74% of the strains from hospitalized patients clustered in genogroup C.

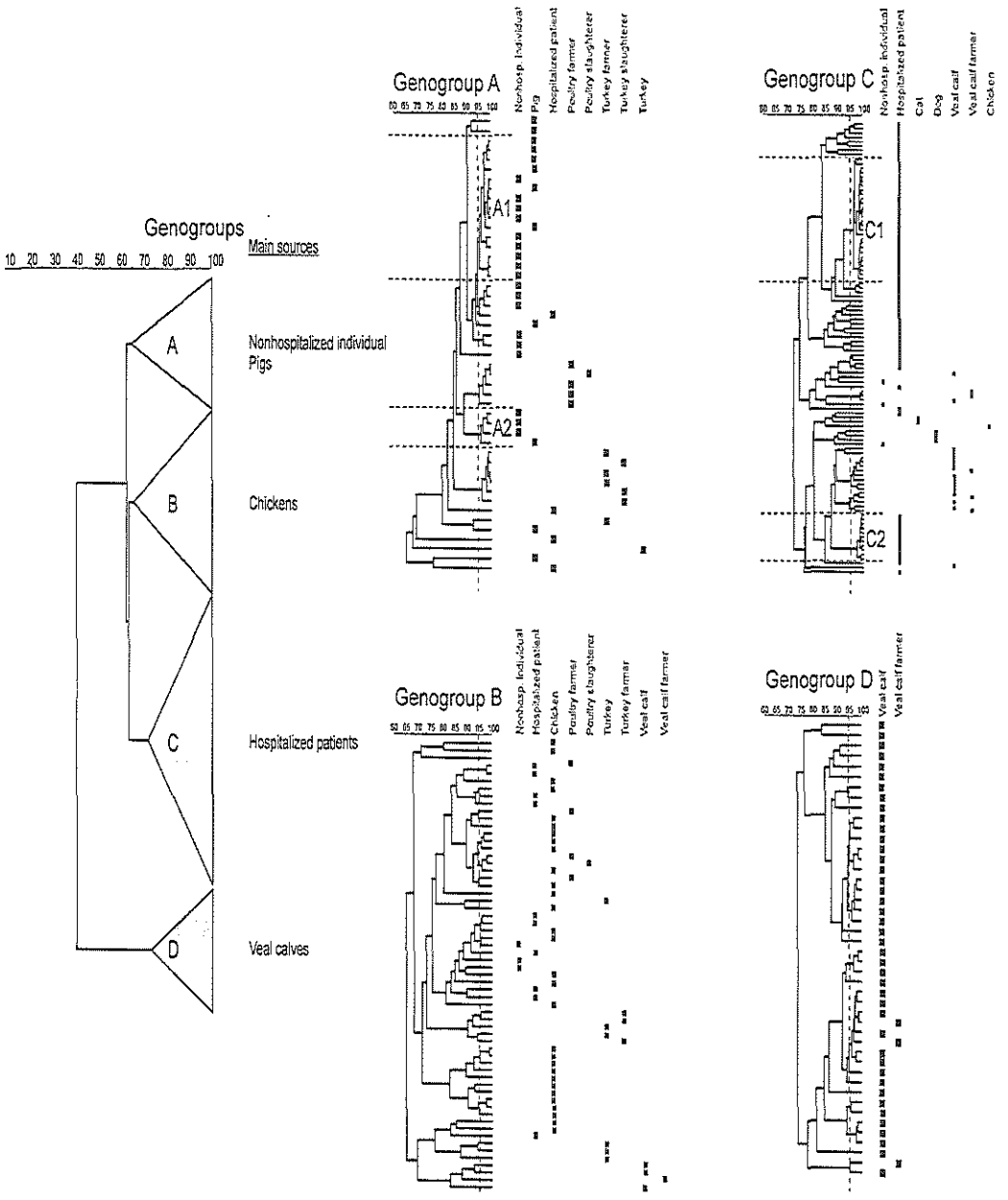


Figure 2: *Abridged dendrogram of all vancomycin-resistant Enterococcus faecium (VREF) strains and separate dendrograms of strains belonging to each of 4 genogroups. Symbols depict VREF strains from various sources. A1, A2, C1, and C2 depict subgroups of genogroups A and C. Numbers on horizontal axis indicate % similarities, as determined by Pearson product-moment correlation coefficient. Vertical dashed lines indicate 95% similarity coefficient, above which strains were considered to be of identical amplified-fragment length polymorphism type.*

Group C strains also comprised some isolates from veal calves and all 5 isolates from cats and dogs. No association was found between the body site of the hospitalized patients from which the strains were recovered from and the grouping of the strains (table 1). Unexpectedly, most isolates recovered from the feces of non-hospitalized persons (75%) were grouped in a different genogroup, group A. Furthermore, half the isolates from poultry farmers or slaughterers and 70% of the isolates from turkey farmers or slaughterers were in this group (figures 2, *3*A). All isolates from pigs were also grouped in genogroup A. Two subgroups, A1 and A2, were distinguished, and either subgroup comprised both pig and human isolates indistinguishable by AFLP (similarity >95%). Genogroup B comprised mainly strains originating from poultry: 97% of the chicken and 86% of the turkey isolates. Interestingly, a large percentage of isolates from poultry farmers or slaughterers were found in taxon B, as well (figures 2, *3*A). Finally, genogroup D comprised exclusively strains from veal calves (70% of the calf strains) and veal calf farmers (figures 2, *3*A). About half the genogroup D strains were similar, and the human isolates were identical to >1 of the calf strains.

*Association between AFLP types and T*n1546 *types*

The T*n1546* types of all 255 VREF strains were determined (figure *3*B). The T*n1546* (sub)types A1, A2, and B have been found elsewhere in strains from humans, pigs, and poultry [9, 12, 14, 17, 18, 20, 21, 25]. Strains with these T*n1546* types were found among 3 or among all 4 *E. faecium* genogroups, suggesting horizontal spread of the VanA transposon among genetically different enterococci (figure *3*B). Strains with transposon types C and E were found in half the genogroups. In contrast, 3 transposon types, A3, D, and F, were confined mainly to a single AFLP genogroup, and these strains were also closely associated with specific hosts. Transposon types D and F were found mainly in genogroup C VREF strains from hospitalized patients, suggesting clonal expansion (figure *3*B). Type A3 was restricted to isolates of genogroup D, and these originated from veal calves and veal calf farmers. Remarkably, no A3 transposon types were found in veal calves present in genogroup C. This suggests the existence of 2 separate VREF subtypes in veal calves.

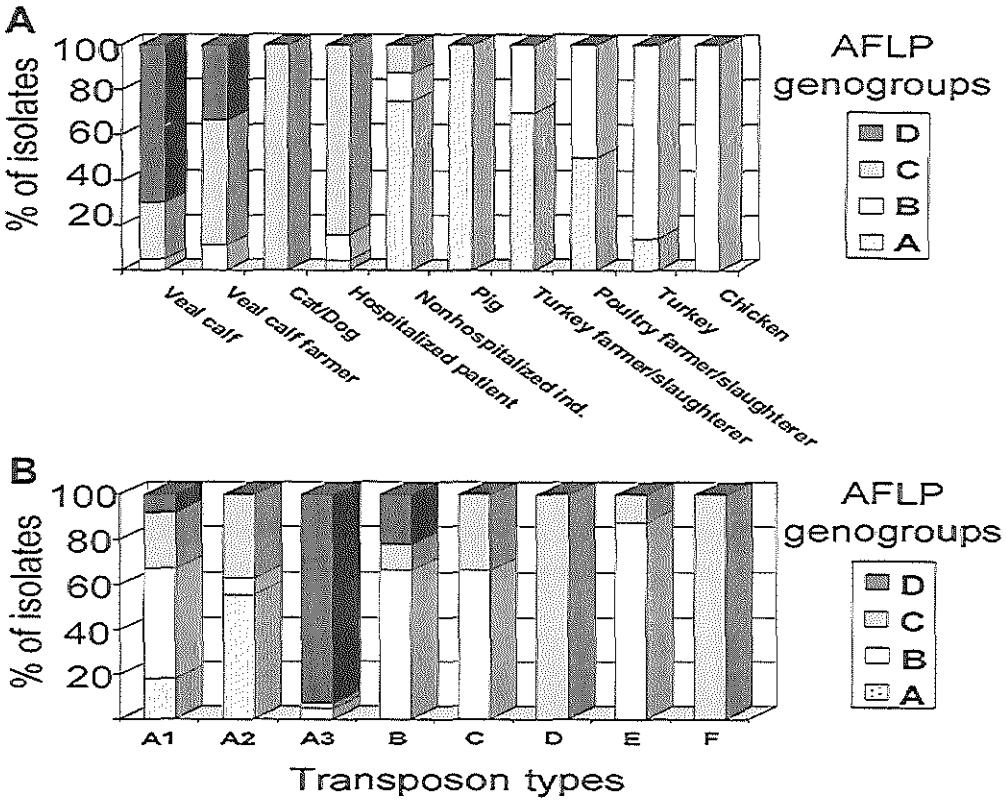


Figure 3: *Distribution of all 255 vancomycin-resistant Enterococcus faecium from various sources among 4 genogroups (A) and distribution of VanA transposon types among 4 genogroups (B). AFLP, amplified-fragment length polymorphism.*

Discussion

This study shows that particular *E. faecium* genogroups are associated with particular hosts and environments, such as farms and hospitals. We limited this study to vancomycin-resistant isolates. Therefore, the results may differ for drug-susceptible isolates. Although many studies of the epidemiology of vancomycin-resistant enterococci have made use of molecular typing, only a few studies have suggested the existence of host-specific ecovars. On the basis of

slight differences in *E. faecium* isolates, Devriese and colleagues [41, 42] have suggested that species-specific variants occur among dogs and poultry. To our knowledge, the study presented here is the first systematic study showing an association between host species and VREF strain genogroup. The disclosure of such ecovars has probably been hampered by the use of typing methods that were too limited in the degree of strain differentiation, such as ribotyping [32, 34], or by the use of methods, such as PFGE, that discriminate well but do not adequately establish the degree of genetic relatedness between dissimilar strains [34, 35, 38]. The AFLP method used in this study permits a high degree of strain differentiation, because of the large number of restriction fragments analyzed and the establishment of genetic relatedness among dissimilar, non–epidemiologically related strains made possible by the presence of shared restriction fragments of evolutionarily more-conserved DNA stretches. Comparison of AFLP with PFGE for 25 VREF isolates, including VREF strains isolated during a hospital outbreak of VREF infections, revealed that the degree of strain differentiation and the identification of outbreak strains by AFLP typing is com-parable with that of PFGE typing. Four distinct genogroups among 255 VREF isolates were disclosed in this study, and strains within each group shared two-thirds or more of their restriction fragments. The use of restriction enzyme combinations other than *Eco*RI-*Cfo*I led to a similar grouping (R.J.L.Willems, unpublished data). The strongest association between host and genogroup was found among strains of genogroup D: virtually all genogroup D strains were from calves and a few were from veal calf farmers. Therefore, the host range of strains of this type is restricted mainly to calves. Although the host range of strains from the remaining 3 genogroups seems broader, these also exhibited a strong association with the source. Strains from chickens and turkeys were found almost exclusively in genogroup B, all pig strains were found in genogroup A, and all 5 strains from cats and dogs were found in genogroup C. Recently, Van den Braak et al. [12] distinguished poultry-specific PFGE types that were not found in humans. The most unexpected finding in this study is the apparent dichotomy between VREF strains isolated from non-hospitalized persons and those isolated from hospitalized patients. The tight genetic clustering of strains from hospitalized patients is even more surprising because these strains were collected from geographically diverse locations (Europe, Israel, and the United States). The strains isolated from hospitalized patients were clustered mainly in

genogroup C, whereas those isolated from non-hospitalized persons without VREF infection were mainly of genogroup A. This difference cannot easily be explained byonly a difference in the habitat in the human body, because we found no significant difference in the distribution among the genogroups of hospital strains from fecal origin or from other infected body sites, such as blood or urine (table 1). A possible explanation for the observed dichotomy is that, in the hospital environment, a subset of VREF strains is being selected that is normally present in low numbers in the human gut. Although the nature of this selective force is not known, it may involve the selection of strains that are more resistant to antibiotics or the selection of strains with specific virulence traits. Various studies suggest that bacteriocins, cytolysins, and hemolysins are more prevalent among enterococci from infected hospitalized patients than among fecal isolates from healthy persons [59–61]. Furthermore, most of the *E. faecalis* strains harboring the putative virulence gene *esp,* which encodes a surface antigen, are infection-derived [62]. It is unknown, however, whether *esp*-positive strains belong to a genetically well-defined taxon, such as the *E. faecium* genogroup C disclosed in this study. Interestingly, all isolates from cats and dogs and 25% of the veal calf isolates grouped in genogroup C, which may suggest that pet animals and veal calves are a potential source of VREF strains for hospitalized patients.

Molecular typing of T*n1546* in the VREF strains analyzed in this study shows that the various VanA transposon variants are not randomly distributed among the 4 main VREF genogroups identified. The transposon types A3, D, E, and F are predominantly found in only 1 genogroup, thus exhibiting a high degree of host specificity. Transposon types A1, A2, and B seem to be more promiscuous, because these are found in most VREF genotypes. This finding confirms our previous findings. In previous studies, types D and F transposons have been found only in hospitalized patients in the United Kingdom and United States, respectively [14], and the E type transposons were found predominantly in poultry [9, 14]. In contrast, types A1, A2, and B transposons were found in various animals, humans, and other sources, including non-hospitalized persons, hospitalized patients, pigs, veal calves, chickens, sewage, turkeys, turkey farmers or slaughterers, a duck, and a pony. One of the aims of the present study and our previous studies [9, 14] was the assessment of the contribution of animal husbandry to the occurrence of vancomycin resistance in humans. In the

fecal flora of poultry farmers and slaughterers and veal calf farmers, we found VREF genotypes that were specific for the animal hosts—poultry and veal calves, respectively. This suggests that VREF strains from animals are transmitted to humans. Because strains of genotypes B and D were not or only rarely found among the general population, these animal-specific strains may colonize humans only transiently. This is in contrast to strains of genotype A. All pig strains were of this genogroup, and most strains from non-hospitalized persons were also of genogroup A. Furthermore, various pig strains were indistinguishable by AFLP from human strains. These data strongly suggest that, in the community, VREF strains in hu-mans mainly originate from pigs in which a high level of glycopeptide resistance has been observed [2, 11, 15]. This idea is consistent with observations by others that humans and pigs may harbor VREF strains with identical PFGE types [17, 26]. The predominance of piglike VREF strains among humans in the community is the result of exposure and survival in the gut. It is presently unclear which of these factors is critical in the ecology of VREF. Thus far, molecular comparison of human- and pig-derived enterococci has been done only on VREF strains. Therefore, further studies also including drug-susceptible enterococci are needed to analyze more extensively the populations of the pre-dominant flora in humans, pigs, and other animals.

Acknowledgments

We thank Marinus Schouten, Wilma Kraak, and Andreas Voss (University Hospital St Radboud, Nijmegen, the Netherlands) for providing clinical isolates from various European countries; Michael Dunne, Jr. (Henry Ford Health System, Detroit) for providing clinical isolates from the United States; J. Zoe Jordens (John Radcliffe Hospital, Oxford, UK) for providing clinical isolates from the United Kingdom; Ans van Veen-Rutgers and Marga van Santen-Verheuvel (National Institute of Public Health and the Environment, Bilthoven, the Neth-erlands) for technical support; and Han de Neeling for helpful discussions and critical review.

References

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

2. Aarestrup FM, Ahrens P, Madsen M, Pallesen LV, Poulsen RL, Westh H: 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 (1996) 40:1938–1940.
3. Bais RK, Freundlich LF, Currie BP: Outpatient prevalence of vancomycin-resistant enterococcal (VRE) enteric colonization in the catchment area of a hospital hyperendemic for VRE [abstract]. Infect Control Hosp Epidemiol (1996) 17(pt 2):P20.
4. Bates J, Jordens JZ, Griffiths DT: Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J Antimicrob Chemother (1994) 34:507–514.
5. Coque TM, Tomayko JF, Ricke SC, Okhyusen PC, Murray BE: Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob Agents Chemother (1996) 40:2605–2609.
6. Devriese LA, Ieven M, Goossens H, et al: Presence of vancomycin-resistant enterococci in farm and pet animals. Antimicrob Agents Chemother (1996) 40:2285-2287.
7. Endtz H, van den Braak N, van Belkum A, et al: Fecal carriage of vancomycin- resistant enterococci in hospitalized patients and those living in the community in the Netherlands. J Clin Microbiol (1997) 35:3026–3031.
8. Klare I, Heier H, Claus H, et al: *Enterococcus faecium* strains with *van*A-mediated high level glycopeptide resistance isolated from animal food-stuffs and fecal samples of humans in the community. Microb Drug Resist (1995) 1:265–272.
9. Stobberingh E, Van Den Bogaard A, London N, Driessen C, Top J, Willems R: Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of the Netherlands: evidence for transmission of vancomycin resistance from animals to humans? Antimicrob Agents Chemother (1999) 43:2215–2221.
10. Thal LA, Chow JW, Mahayni R, et al: Characterization of antimicrobial resistance in enterococci of animal origin. Antimicrob Agents Chemother (1995) 39:2112–2115.
11. Van den Bogaard AE, Mertens P, London NH, Stobberingh EE: High prevalence of colonization with vancomycin- and pristinamycin-resistant enterococci in healthy humans and pigs in the Netherlands: is the addition of antibiotics to animal feeds to blame? J Antimicrob Chemother (1997) 40:454–456.
12. Van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HP: Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in the Netherlands. J Clin Microbiol (1998) 36:1927–1932.
13. Van der Auwera P, Pensart N, Korten V, Murray BE, Leclercq R: Influence of oral glycopeptides on the fecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J Infect Dis (1996) 173:1129–1136.
14. Willems RJL, Top J, Van Den Braak N, et al: Molecular diversity and evo-lutionary relationships of T*n1546*-like elements in enterococci from hu-mans and animals. Antimicrob Agents Chemother (1999) 43:483–491.
15. Bager F, Madsen M, Christensen J, Aarestrup FM: Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev Vet Med (1997) 31:95–112.
16. Arthur M, Molinas C, Depardieu F, Courvalin P: Characterization of T*n1546*, a T*n*3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol (1993) 175:117–127.
17. Descheemaeker PRM, Chapelle S, Devriese LA, Butaye P, Vandamme P, Goossens H: Comparison of glycopeptide-resistant *Enterococcus faecium* isolates and glycopeptide resistance genes of human and animal origins. Antimicrob Agents Chemother (1999) 43:2032–2037.
18. Jensen LB: Differences in the occurrence of two base pair variants of T*n1546* from vancomycin-resistant enterococci from humans, pigs, and poultry. Antimicrob Agents Chemother (1998) 42:2463–2464.

19. Jensen LB: Internal size variations in *Tn1546*-like elements due to the presence of *IS1216V.* FEMS Microbiol Lett (1998) 169:349–354.
20. Jensen LB, Ahrens P, Dons L, Jones RN, Hammerum AM, Aarestrup FM: Molecular analysis of *Tn1546* in *Enterococcus faecium* isolated from animals and humans. J Clin Microbiol (1998) 36:437–442.
21. Palepou MFI, Adebiyi AMA, Tremlett CH, Jensen LB, Woodford N: Molecular analysis of diverse elements mediating VanA glycopeptide resistance in enterococci. J Antimicrob Chemother (1998) 42:605–612.
22. Simonsen GS, Haaheim H, Dahl KH, et al: Transmission of VanA-type vancomycin-resistant enterococci and *vanA* resistance elements between chicken and humans at avoparcin-exposed farms. Microb Drug Resist (1998) 4:313–318.
23. Van den Bogaard AE, Jensen LB, Stobberingh EE: Vancomycin-resistant enterococci in turkeys and farmers. N Engl J Med (1997) 337:1558–1159.
24. Werner G, Klare I, Witte W: Arrangement of the *vanA* gene cluster in en-terococci of different ecological origin. FEMS Microbiol Lett (1997) 155:55–61.
25. Woodford N, Adebiyi AMA, Palepou MFI, Cookson BD: Diversity of VanA glycopeptide resistance elements in enterococci from humans and non-human sources. Antimicrob Agents Chemother (1998) 42:502–508.
26. Jensen LB, Hammerum AM, Poulsen RL, Westh H: Vancomycin-resistant *Enterococcus faecium* strains with highly similar pulsed-field gel electrophoresis patterns containing similar *Tn1546*-like elements isolated from a hospitalized patient and pigs in Denmark. Antimicrob Agents Chemother (1999) 43:724–725.
27. Robredo B, Singh KV, Torres C, Murray BE: Evidence by PFGE of colonization of animals and humans by common strains of *vanA* containing enterococci [abstract LB2]. In: Program and abstracts of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Diego). Washington, DC: American Society for Microbiology, 1998.
28. van Belkum A, van den Braak N, Thomassen R, Verbrugh H, Endtz H: Vancomycin-resistant enterococci in cats and dogs. Lancet (1996) 348:1038–1039.
29. Berchieri A: Intestinal colonization of a human subject by vancomycin-resistant *Enterococcus faecium.* Clin Microbiol Infect (1999) 5:97–100.
30. Barbier N, Saulnier P, Chachaty E, Dumontier S, Andremont A: Random amplified polymorphic DNA typing versus pulsed field gel electrophoresis for epidemiological typing of vancomycin-resistant enterococci. J Clin Microbiol (1996) 34:1096–1099.
31. Descheemaeker P, Lammens C, Pot B, Vandamme P, Goossens H: Evaluation of arbitrarily primed PCR analysis and pulsed-field gel electrophoresis of large genomic DNA fragments for identification of enterococci important in human medicine. Int J Syst Bacteriol (1997) 47:555–561.
32. Gordillo ME, Singh KV, Murray BE: Comparison of ribotyping and pulsed-field gel electrophoresis for subspecies differentiation of strains of *Enterococcus faecalis.* J Clin Microbiol (1993) 31:1570–1574.
33. Issack MI, Power EGM, French GL: Investigation of an outbreak of van-comycin- resistant *Enterococcus faecium* by random amplified polymorphic DNA (RAPD) assay. J Hosp Infect (1996) 33:191–200.
34. Kuhn I, Burman LG, Haeggman S, Tullus K, Murray BE: Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. J Clin Microbiol (1995) 33:2812–2817.
35. Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD: DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. J Clin Microbiol (1999) 37:1084–1091.
36. Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM: Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J Clin Microbiol (1990) 28: 2059–2063.
37. Quednau M, Ahrne S, Molin G: Genomic relationships between *Enterococcus faecium* strains from different sources and with different antibiotic resistance profiles evaluated by restriction endonuclease analysis of total chromosomal DNA using *Eco*RI and *Pvu*II. Appl Environ Microbiol (1999) 65:1777–1780.

38. Savor C, Pfaller MA, Kruszynski JA, Hollis RJ, Noskin GA, Peterson LR: Comparison of genomic methods for differentiating strains of *Enterococcus faecium:* assessment using clinical epidemiologic data. J Clin Microbiol (1998) 36:3327–3331.
39. Sechi LA, Zanetti S, Dupre I, et al: Molecular epidemiology by ribotyping and PCR-ribotyping of *Enterococcus faecium* strains isolated from intercontinental areas. New Microbiol (1998) 21:113–122.
40. Tomayko JF, Murray BE: Analysis of *Enterococcus faecalis* isolates from intercontinental sources by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis. J Clin Microbiol (1995) 33:2903–2907.
41. Devriese LA, Cruz Colque JI, De Herdt P, Haesebrouck F: Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. J Appl Bacteriol (1992) 73:421–425.
42. Devriese LA, Van de Kerckhove A, Kilpper-Balz R, Schleifer KH: Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int J Sys Bacteriol (1987) 37:257–259.
43. Vos P, Hogers R, Bleeker M, et al: AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res (1995) 23:4407–14.
44. Bootsma HJ, van der Heide HGJ, van de Pas S, Schouls LM, Mooi FR: Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. J Infect Dis (2000) 181:1376–1387.
45. Arnold C, Metherell L, Willshaw G, Maggs A, Stanley J: Predictive fluorescent amplified fragment length polymorphism analysis of *Escherichia coli:* high-resolution typing method with phylogenetic significance. J Clin Microbiol (1999) 37:1274–1279.
46. Desai M, Efstratiou A, George R, Stanley J: High-resolution genotyping of *Streptococcus pyogenes* serotype m1 isolates by fluorescent amplified fragment length polymorphism analysis. J Clin Microbiol (1999) 37:1948–1952.
47. Desai M, Tanna A, Wall R, Efstratiou A, George R, Stanley J: Fluorescent amplified fragment length polymorphism analysis of an outbreak of group A streptococcal invasive disease. J Clin Microbiol (1998) 36:3133–3137.
48. Duim B, Wassenaar TM, Rigter A, Wagenaar J: High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl Environ Microbiol (1999) 65:2369–2375.
49. Grady R, Desai M, O'Neill G, Cookson B, Stanley J: Genotyping of epidemic methicillin-resistant *Staphylococcus aureus* phage type 15 isolates by fluo-rescent amplified fragment length polymorphism analysis. J Clin Micro-biol (1999) 37:3198–3203.
50. Kokotovic B, Friis NF, Jensen JS, Ahrens P: Amplified fragment length polymorphism fingerprinting of *Mycoplasma* species. J Clin Microbiol (1999) 37:3300–3307.
51. Savelkoul PHM, Aarts HJM, De Haas J, et al: Amplified fragment length polymorphism analysis: the state of an art. J Clin Microbiol (1999) 37: 3083–3091.
52. Van Eldere J, Janssen P, Hoefnagels Schuermans A, Van Lierde S, Peetermans WE, Quality Control Study Group: Amplified fragment length polymorphism analysis versus macro-restriction fragment analysis for molecular typing of *Streptococcus pneumoniae* isolates. J Clin Microbiol (1999) 37: 2053–2057.
53. Bonten MJM, Hayden MK, Nathan C, et al: Epidemiology of colonisation of patients and environment with vancomycin-resistant enterococci. Lancet (1996) 348:1615–1619.
54. Dunne WM, Wang W: Clonal dissemination and colony morphotype variation of vancomycin-resistant *Enterococcus faecium* isolates in metropolitan Detroit, Michigan. J Clin Microbiol (1997) 35:388–392.
55. Jordens JZ, Bates J, Griffiths DT: Faecal carriage and nosocomial spread of vancomycin-resistant *Enterococcus faecium.* J Antimicrob Chemother (1994) 34:515–528.
56. Leclercq R, Derlot E, Weber M, Duval J, Courvalin P: Transferable vanco-mycin and teicoplanin resistance in *Enterococcus faecium.* Antimicrob Agents Chemother (1989) 33:10–15.

57. Schouten MA, Voss A, Hoogkamp Korstanje JAA, European VRE Study Group: Antimicrobial susceptibility patterns of enterococci causing infections in Europe. Antimicrob Agents Chemother (1999) 43:2542–2546.
58. van den Bogaard AEJM, London N, Stobberingh EE: Antimicrobial resistance in pig faecal samples from The Netherlands (five abattoirs) and Sweden. J Antimicrob Chemother (2000) 45:663–671.
59. Coque TM, Patterson JE, Steckelberg JM, Murray BE: Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J Infect Dis (1995) 171:1223–1229.
60. Del Campo R, Tenorio C, Robredo B, Diez C, Baquero F, Gomez-Lus RTC: Bacteriocin production in *Enterococcus* spp. from different origins [abstract C-95]. In: Program and abstracts of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Diego). Washington, DC: American Society for Microbiology, (1998) 96.
61. Haas W, Gilmore MS. Molecular nature of a novel bacterial toxin: the cytolysin of *Enterococcus faecalis.* Med Microbiol Immunol (1999) 187:183–190.
62. Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS: Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infect Immun (1999) 67:193–200.

CHAPTER 11

General Discussion

Enterococci harboring the *vanA* gene are high-level vancomycin and teicoplanin resistant and can cause serious infections that are hard to treat. The resistance is transferable to other bacteria and treatment of the infection is only effective when combining antimicrobials or using experimental antimicrobial agents that have as yet unproven efficacy. Lack of detection and control can result in endemic occurrence of the organisms. The prevalence of vancomycin-resistant enterococci (VRE) has risen alarmingly over the last decades, especially in hospitals. Consequently, researchers all over the world have started to investigate its dissemination, risk factors for acquisition and virulence. In the USA, the lack of a balanced antibiotic policy is thought to be one of the reasons for this increase of VRE-related clinical problems. In 1995, the Hospital Infection Control Practices Advisory Committee (HICPAC) presented recommendations for preventing and controlling the spread of vancomycin resistance [1]. The HICPAC advised prudent use of vancomycin and related antimicrobials by clinicians, education of hospital personal, thereby preventing person-to-person transmission and, last but not least, early detection and rapid reporting of vancomycin resistance in enterococci and other Gram- positive bacteria. Consequently, the microbiology laboratory became part of the first line of defense against the spread of VRE.

Tentative Dutch guidelines for VRE control have been written recently, as the VRE epidemiology in Europe differs significantly from that in the United States [2, 3]. In Europe the prevalence of VRE is low and VRE outbreaks are usually not part of an endemic problem. The "search and destroy" policy which is used for MRSA control in The Netherlands is not effective in VRE since, in contrast to MRSA colonization, VRE colonization is not restricted to the hospital setting in Europe. Both Bonten and Schouten have suggested that extensive measures should only be implemented when dissemination of one single genotype of VRE is demonstrated to prevent further spread of the VRE strain in the hospital [2, 3].

Since timely and accurate detection of VRE is mandatory to prevent strain dissemination, the work described in this thesis was focussed in molecular and microbiological approaches of the identification and typing of VRE and their resistance determinant *Tn1546*.

Detection of VRE

Accurate and rapid detection of vancomycin resistance is essential in any strategy that aims to prevent nosocomial transmission of these resistant organisms. Although new automated technology for the detection of antimicrobial resistance in bacteria has been developed, it is known that some vancomycin resistance phenotypes are difficult to detect accurately.

In order to evaluate test efficacy, comparative analyses of VRE identification assays are warranted. When several of these tests, such as agar dilution, disc diffusion, E-test, vancomycin screen agar plate, Microscan® (conventional and rapid panel), VITEK®1 (GPS-TA and GPS-101 cards), and VITEK®2, were compared, no major failures in detecting *vanA* enterococci were demonstrated (Chapter 2 and 3). For identification and susceptibility testing, most conventional methods require a full 24 h of incubation, whereas automated methods significantly reduce the time to VRE detection. Earlier detection of resistant bacteria allows for faster alerting of the hospital staff, who can then take precautionary measures. It has been demonstrated that this type of rapid reporting of identification and susceptibility results may have important benefits in terms of patient outcome and cost effectiveness [4]. In contrast to *vanA* mediated resistance, we found that automated detection of *vanB* and *vanC* enterococci remains difficult. These results are in concordance with other recent studies that have evaluated commercial methods for susceptibility testing of vancomycin resistant enterococci [5,6]. However, improvement of the performance of the automated tests, as shown for the Vitek1 GPS-101 card and the new VITEK®2 system, have led to increased detection rates of *vanB* and *vanC* enterococci.

The prevalence and clinical implications of *E. gallinarum (vanC1)* and *E. casseliflavus (vanC2)* is still unknown. However, recently some reports of clinically significant infections with *vanC* enterococci have been published [9]. These latter VRE species are often misidentified by current diagnostic systems and their intermediate level of resistance may go unrecognized. It is thus likely that infections by these two *vanC* VRE species are underreported in the literature. Recently, several studies have compared different methods for the identification of enterococci harboring the *vanC* gene. They conclude that methyl-α-D-glycopyranoside fermentation and a few other biochemical assays

are simple, accurate and less costly tests for the identification of these species [7, 8].

With respect to rapid and fully automated methods, we found the agar screen the most reliable and easy-to-perform method for routine screening, especially when detection of *vanA-, vanB-,* and *vanC1/C2*-mediated resistance in enterococci is required. In another recent study we reported the lack of commercial susceptibility testing methods to differentiate hetero-glycopeptide intermediate *Staphylococcus* species from glycopeptide susceptible strains [10]. It is clear that further improvement of test speed without compromising the accuracy to detect all forms of vancomycin resistance in enterococci and other Gram-positive cocci is still needed.

Prevalence of VRE in (non-)hospitalized patients in The Netherlands

Several studies have described fundamental differences in the epidemiology of vancomycin resistance in the United States and Europe [11, 12]. In Europe there is ample evidence suggesting that VRE were introduced into hospitals by patients already colonized in the community. In contrast in the USA it is unclear how VRE were nosocomially introduced and distributed. It is quite likely that the dramatic increase of vancomycin use in the USA over the past decade has played a crucial role. In chapter 4 and 5 we described the VRE prevalence in (non-) hospitalized patients in The Netherlands. In Dutch hospitals, VRE carriage in adult and pediatric patients on intensive care units and hemato-oncology wards remains low. Using selective enrichment broth for isolation of VRE, in four yearly point prevalence studies between 1995 and 1998, low incidences of VRE present in fecal swabs were observed. It was determined that 55% of all individuals were intestinal carriers of vancomycin susceptible enterococci. Only 1.4% of all ICU and hematology patients included in the study carried VRE. It must be emphasized that in stool samples submitted from out-patients similar VRE carriage rates were found. From 1995-1998, no increase in the VRE colonization rate was observed. Eleven VRE strains were detected and identified as *Enterococcus faecium* and four as *Enterococcus faecalis.* All *E. faecium* and one *E. faecalis* carried the *vanA* gene; the other *E. faecalis* strains harbored the *vanB* gene. PFGE revealed that 3 *vanB* VRE isolated from patients hospitalized in one

single ICU, were genetically related, suggesting nosocomial transmission. In contrast, when analyzing the 11 *E. faecium* isolates we found 4 that were genetically related without any geographical clustering.

The Dutch situation differs from the situation in the USA, where hospitalized patients have been reported to be more frequently colonized, whereas outside of the hospital VRE are thought not to be present in the environment at all. It is interesting to note that the Dutch data are corroborated by other European studies, where a low prevalence has been documented within healthcare settings in the same period. In a study by Schouten et al. the prevalence of VRE carriage in hospitalized patients all over Europe ranged from 0% to 3% [13]. Suprisingly, no vanA or vanB related VRE were isolated in these hospitalized patients in the Netherlands. However, community-drived data sets from neighboring geographic locales may be significantly different. In Belgian, British and other Dutch studies for instance, higher VRE carriage rates of 11%, 12% and 17%, respectively, have been documented [14-16]. In contrast, an extremely high prevalence rate was found among turkey farmers (39%) and slaughterers (20%) in the Netherlands [17]. Overall, we may conclude that various scenarios describing the prevalence of VRE in the community versus that in hospitalized patients are encountered. Levels of colonization can vary and local prevalence can differ greatly. The outcomes of VRE prevalence studies in various countries are difficult to compare as different methodologies for culturing are often used [18]. Thus, the prevalence of VRE in The Netherlands and other European countries remains low. However, a universal standardized protocol for the (laboratory) detection of VRE carriers and/or infection rate in (non-) hospitalized persons is needed to determine the exact prevalence of VRE in each country.

VRE colonization and risk factors

In recent years, various risk-factors for acquisition of VRE colonization and infection have been described. Known risk factors are prolonged hospital stay, severity of illness, renal failure, immunosuppression, recent surgery and prior exposure to antimicrobial agents [19-21]. However, diarrhea, age and location have also been documented as risk for VRE colonization [22, 23]. In contrast to several other studies [20, 21] we reported in chapter 5 that VRE carriage is not associated with prolonged hospital stay in The Netherlands. Other studies

showed results similair to our findings and also did not find prolonged hospital stay to be a risk factor of VRE colonization [24, 25]. It is important to mention that the number of risk factors analyzed in our study was limited, and did not include, for example, analysis of factors such as previous antimicrobial therapy or proximity to known patients with VRE. Therefore, more studies in The Netherlands are needed to further clarify the epidemiology of VRE in order to design of future interventions.

VRE and the environment including zoonoses

Of major European concern is the apparent relationship between the presence of vancomycin resistance in enterococci and the use of avoparcin, a glycopeptide homologue, as a growth-promoting additive in animal food [26]. The addition of antibiotics to animal food has been documented as being economically valid: animals grow faster and reach higher weights [27, 28], consequently, a significant quantity of antibiotics has been used for this purpose. Evidence is accumulating that antibiotics thus introduced into environments where enterococci thrive may have led to the high incidences of VRE in animal husbandry [29].

The influence of avoparcin antibiotics on the selection of intestinal *vanA*-VRE in chickens has recently been studied [30]. The results of this study indicate that avoparcin supplementation increases the selection of VRE. Furthermore, the study we describe in chapter 7 demonstrated the high prevalence of VRE in chicken products at the retail level in The Netherlands. The rate of contamination with VanA VRE in chicken meat was 100% in certain areas, with an overall prevalence of 79% nationwide, 59% of these were high level vancomycin-resistant *Enterococcus faecium* (VREF). PFGE revealed extensive VREF heterogeneity. However, two genotypes were found nationwide on multiple occasions. No PFGE-deduced genetic overlap was found, when VREF from humans were compared with the VREF poultry strains. Two *vanA* transposon types were identified among poultry strains. In 59/142 (42%) of the poultry VREF, the size of the intergenic region between *vanX* and *vanY* was ± 1300 bp. This transposon type was not found in human VREF. In contrast, all human strains and 83/142 (58%) of the poultry VREF contained an intergenic region with the size of 543 bp. Sequencing this 543 bp intergenic *vanX-vanY* region demonstrated full sequence conservation. Though

preliminary, these data suggest that dissemination of the resistance genes encoded on transposable elements may be of greater importance than clonal dissemination of resistant strains.

Subsequently, many similar studies in Europe [31] and USA [32] reported the existence and importance of non-human VRE reservoirs. Frequent strain sharing was documented among turkey farmers and their flocks for instance [17]. This proves that rich environmental and zoonotic VRE sources in the vicinity may well lead to cross-colonization of humans. Even domestic cats and dogs (Chapter 6b) may provide reservoirs. It goes without saying that these sources are "intimately" linked to humans and may thus be important for colonization of the human intestine. A similar and equally worrisome association can be seen in the case of avilamycin, an oligosaccharide antibiotic that is also used as a food additive [33]. Again, resistance traits can be detected in a significant proportion of enterococcal strains isolated from farm animals. Use of the new "human homologue" of avilamycin, everninomycin, may well lead to the selection of similar mutants in the clinical setting, particularly in the hospital where such new generations of antibiotics are initially evaluated.

In contrast, in countries were avoparcin has not been used in animal husbandry, hardly any VRE were detected in healthy humans, animals or animal related food products [34]. Due to this observation the European Commission decided in April 1997 to ban the use of avorpacin as growth promoter all over Europe. Van den Bogaard et al. described the effect of banning avoparcin in The Netherlands in 2000. The prevalence of VRE in humans, broilers and pigs decreased significantly within two years after the ban. In other European countries such as Denmark, Germany and Italy the same effect was observed. Apparently, banning veterinary uses of glycopetide analogue results in elimination of important sources of VRE and, consequently, may lead to a lesser burden in human medicine as well. However, the presence of VRE is not only restricted to the bio-industry, since VRE has been detected in various other animals and in the environment. The question arises whether dissemination of VRE is now so extensive that elimination of all VRE is impossible.

VRE and dietary habits

One way of examining the relationship between contamination of the environmental with VRE to human VRE carriership is to measure the effect of VRE contamination of meat products by the individual meat consumer. The prevalence of VRE in vegetarians versus that in non-vegetarians could be illustrative in this respect. The results of such studies have been published recently and suggested a close relationship between dietary habits and the presence of VRE in the gut flora. Microbiological studies performed on faecal specimens obtained from people living in an old people's home for vegetarians and from non-vegetarian controls from the same age group, showed statistically significant differences in carriage rates of *vanA* containing VRE [35]. Among the vegetarians, the VRE colonization rate tended to be lower although statistical significance was not reached. Our study described in chapter 6a provided evidence for another colonization scenario. We did not observe differences in colonization with *vanA* or *vanB* VRE between vegetarians and non-vegetarian controls. However, vegetarians were more often colonized with VanC enterococci. One possible explanation would be that *VanC* VRE in particular, are known to colonize plants rather than animals. Both studies suggest a relationship between dietary habits and intestinal colonization by enterococci with reduced susceptibility to glycopeptide antibiotics. Recently, Blom et al. [36] described a randomized double-blind study on ingestion of VRE strains of poultry origin by human healthy volunteers and subsequent follow-up on VRE carriage in these individuals. Two weeks after ingestion (10^7 CFU VRE) no VRE were found in the faeces of these persons, suggesting that VRE from poultry origin do not easily colonize the human intestinal tract. This raises the question whether or not dietary habits influence the colonization rate with respect to enterococci in general.

Molecular typing of VRE

The last decennia several molecular typing techniques, such as DNA restriction fragment analysis, total plasmid profile analysis, Random Amplified Polymorhic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and ribotyping, have been used for epidemiological investigations of enterococcal outbreaks and for

subtyping of enterococcal strains [37]. Use of these techniques leads to enhanced insight in the spread of bacteria. Every single technique is more or less suitable for genotyping of bacterial strains. In chapter 4 and 8 we compared PFGE and RAPD for epidemiological typing of VRE of vancomycin resistant enterococci. PFGE is the current gold standard for molecular analysis for several bacterial species. The advantage of this technique is its high discriminatory power and reproducibility. However, a minimum of 4 days is required before results are available. Moreover, expensive enzymes and special instruments are needed. Recently an improved, rapid and potentially cost-effective PFGE typing method for VRE has been reported [37]. RAPD is known as a fast and simple technique with somewhat less discriminating power and reproducibility. In our studies it appeared that the results of both typing techniques were concordant. Discrepancies between both techniques occur, but overall fingerprint clustering leads to comparable results. In conclusion, to reduce time and costs, RAPD is an appropriate technique to use for elucidating local spread of VRE. However, in cases of large clonal outbreaks of VRE we recommend PFGE, because of the high degree of strain differentiation with this technique and the possibility to exchange data between laboratories. Due to its high discriminatory power, however, PFGE is less suitable in verifying the degree of genetic relatedness between epidemiologically un-related strains, because the banding patterns of such strains are generally completely distinct. Amplified Fragment Length Polymorphism (AFLP) is a high-resolution genotyping method [38]. This method has been applied for typing of several other micro-organisms and has the advantage of high levels of discriminatory power, reproducibility, and standardization [39-42]. For AFLP analysis, restriction fragments of chromosomal DNA are selectively amplified by PCR. By using primers containing one or more selective nucleotides extending at the 3' ends, only a subset of fragments is amplified under stringent PCR conditions. AFLP is capable of establishing a degree of genetic relatedness between strains that by PFGE show no such relatedness. In chapter 10 we used AFLP to determine the genetic relationship among 255 VRE strains derived from diverse sources. *E. faecium* strains from hospitalized patients, healthy human and strains from various animal sources isolated from England, USA and The Netherlands were analyzed to investigate host-specificity of VRE. Among these strains we could discriminate four major AFLP genogroups. Strains of each group shared more than 65% of the amplified

restriction fragments. Genogroup A contained 75% of the strains isolated from non-hospitalized patients and all pig derived isolates. Ninety seven percent of the chicken isolates and 86% of turkey VRE were clustered in group B, whereas 25% of strains derived from veal calves, 84% of hospitalized patients isolates and all dog- and cat- derived VRE gathered in group C. The last group (Genogroup D) mostly contained veal calve isolates (70%). We discovered that vancomycin resistant *E. faecium* strains are mainly host-restricted and that isolates from hospitalized patients do not mix genetically with strains from non-hospitalized patients. However, VRE isolates from pig origin clustered with strains derived from non-hospitalized patients. Species-restricted variants of VRE have been described earlier by Devriese et al.; they described species that occur among dogs and poultry [43-44] and in chapter 7 we distinguished poultry VRE with specific PFGE types that were not found in humans.

In conclusion, this study provides evidence that some animals more than others may play an important role in dissemination of VRE to humans in The Netherlands. Further investigations are necessary to clarify the exact dissemination and the epidemiology of VRE.

Heterogeneity in the resistance mediating genetic element

We analyzed the *Tn1546* transposon present in a large collection of VRE isolates to gain more insight in the evolution of this specific resistant element. In chapter 9 we genetically characterized *Tn1546* and found twenty-two structural variants of this transposon. Furthermore, a hypothetical evolutionary scheme was made for the various *Tn1546* derivatives and we suggested that horizontal transmission of *Tn1546* transposon might be an alternative determinant factor driving the spread of vancomycin resistance. Transposon polymorphism has been demonstrated for the first time by Handwerger et al. [45]. More recently, two studies on the determination of genetic polymorphism in *Tn1546* have been published [46, 47]. A common finding of these latter studies was that although coding potentials remain generally unaffected, transposon polymorphism could be detected quite easily. The presence of additional insertion elements in intergenic positions or deletions at the transposon termini is encountered frequently. This allows adequate tracking of transposon types, which has already demonstrated that certain types can be encountered among VRE from both

human and nonhuman sources. On the other hand, "source-specific" transposons were identified as well. In view of these data research on horizontal gene transfer and detection of common reservoirs for glycopeptide resistance should be initiated. Furthermore, the structure-function relationship of transposons can be assessed: preliminary data suggest that mutations in the transposon may correlate with lowered conjugative potential. An important conclusion that can be drawn from the genome and transposon scanning data is that several epidemiologic scenarios can be envisaged. Epidemic VRE can be identified; in the UK, for instance, various examples of strains that seem to have traveled large distances are acknowledged [48]. Certain VRE are capable of highly efficient bacterial transfer from patient to patient in the hospital environment [49], whereas long-term colonization with a single type of VRE in a single individual has been observed as well [50]. In contrast, in certain hospital settings highly promiscuous transposon types have been described. Instead of epidemic spread of a strain, a specific *Tn1546* type is encountered in various VRE genomes. In conclusion, the spread of vancomycin resistance is facilitated by the epidemic capacity of both strains and transposons. In combination with the large number of environmental and animal reservoirs, at least in Europe, that have already been identified to date, this means that the clinical threat posed by VRE may not be easily overcome in the near future.

Concluding remarks

The prevalence of VRE in hospitalized patients on Intensive-Care-, hematology-oncology- and hemodialysis wards in Dutch hospitals remains low in the period between 1995 and 1998. In 1999 and 2000, however, three outbreaks of VRE were discovered in hospital settings in Amsterdam, Utrecht and Amersfoort [51-53]. Although in each of these outbreaks the number of patients with infections was low, the spread of the colonization rate was high. In each of these events it was possible to stop the spread of VRE and finally eliminate the resistant bacteria from the hospital. The high incidence of VRE in animal husbandry and meat products in The Netherlands may lead to transmission of VRE to humans in and outside the hospital. However, the ban of avoparcin has been followed by a decrease of colonization of VRE in farm animals and non-hospitalized persons [34]. Discussions on the best way to prevent the spread of

VRE in hospital settings are still ongoing [2, 3]. We suggested that horizontal transmission of *Tn1546* transposon might be an alternative determinant factor driving the spread of vancomycin resistance. This scenario rents on an epidemic transposon rather than on patient-to-patient transfer of a given VRE strain. Even at the time of an ongoing outbreak, VRE can change its genotype, since in individual patients the transfer of *Tn1546* to previously vancomycin-susceptible *Enterococcus* strains has been observed to occur in vivo. Spread of vancomycin resistance , therefore, may not be confined to the spread of resistant strains. Clearly, further investigations are needed to gain more detailed insight and to prevent spread of infections caused by vancomycin resistant enterococci. For instance it was recently shown that in all non epidemic and animal VRE isolates a variant of the *esp* gene was absent, interestingly all investigated epidemic VRE strains contained the gene [54].

References

1. Hospital Infection Control Practices Advisory Committee: Recommendation for preventing the spread of vancomycin resistance: Recommendation of the Hospital Infection Control Practices Advisory Committee (HICPAC). Am. J. Infect. Control (1995) 23:87-94.
2. Bonten MJM, Mascini EM, Willem R, Timmer GJ, Gaillard CA, Vandenbroucke-Grauls CMJE: Wat te doen als men vancomycine resistente enterococcen aantreft? Ned. Tijdschr. Geneeskd. (2000) 53:2545-2549.
3. Schouten MA, Hoogkamp-Korstanje JAA, Voss A: Risk factors for VRE acquisition and guidelines for VRE control. Thesis Vancomycin resistant enterococci: epidemiology and control (2000) chapter 8.2: 98-111.
4. Barenfanger J, Drake C, Kacich G: Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. J.Clin. Microbiol. (1999) 37:1415-1418.
5. Okabe T, Oana K, Kawakami Y, Yamaguchi M, Takahashi Y, Okimura Y, Honda T, Katsyuma T: Limitations of Vitek GPS-418 cards in exact detection of vancomycin-resistant enterococci with the *vanB* genotype. J. Clin. Microbiol. (2000) 38:2409-2411.
6. Williams-Bouyer N, Reisner BS, Woodmansee CE, Falk PS, Mayhall CG: Comparison of the Vitek GPS-TB card with disk diffusion testing for predicting the susceptibility of enterococci to vancomycin. Arch. Pathol. Lab. Med. (1999) 123:622-625.
7. Devriese LA, Pot B, Kersters K, Lauwers S, Haesebrouck F: Acidification of Methyl- -D-Glucopyranidose: a useful test to differentiate *Enterococcus casseliflavus* and *Enterococcus gallinarum* from *Enterococcus faecium* species group and from *Enterococcus faecalis*. J. Clin. Microbiol. (1996) 34:2607-2608.
8. Ramotar K, Woods W, Larocque L, Toye B: Comparison of phenotypic methods to identify enterococci intrinsically resistant to vancomycin (VanC VRE). Diagn. Microbiol. Infect. Dis. (2000) 36:119-124.
9. Yoshimoto E, Konishi M, Takahashi K, Majima T, Ueda K, Murakawa K, Sakamoto M, Maeda K, Mikasa K, Narita N, Sano R, Masutani T, Ishii Y, Yamaguchi K: *Enterococcus gallinarum* septicemia in a patient with acute myeloid leukemia. Kansenshogaku Zasshi (1999) 73:1078-1091.
10. Van den Braak N, van Belkum A, te Witt R, Verbrugh HA, Endtz HPh: Glycopeptide resistance in *Staphylococcus* spp. J. Antimicrob. Chemotherp. (1998) 42:673-675.
11. Martone WJ: Spread of vancomycin-resistant enterococci: Why did it happen in the Unites States? Infect. Control. Hosp. Epidemiol. (1998) 19:539-545.

12. Goossens H: Spread of vancomycin-resistant enterococci: differences between the United States and Europe. Infect. Control. Hosp. Epidemiol. (1998) 19:546-550.
13. Schouten MA, Hoogkamp-Korstanje JAA, Meis JFGM, Voss A and the European VRE study group: Prevalence of vancomycin-resistant enterococci (VRE) in Europe. Eur. J. Clin. Microbiol. Inf. Dis. In press.
14. Van den Bogaard AE, Stobbering EE: Time to ban all antibiotics as animal growth promotors? Lancet (1996) 31:619.
15. Nelson RRS, McGregor KF, Brown AR, Amyes SGB, Young HK: Isolation and characterization of glycopeptide-resistant enterococci from hospitalized patients over a 30-month period. J. Clin. Microbiol. (2000) 38:2112-2116.
16. Van der Auwera P, Pensart N, Korten V, Murray BE, Leclerq R: Influence of oral glycopeptides on the faecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J.Infect. Dis (1996) 173:1129-1136.
17. Stobbering E, van den Bogaard A, London N, Driessen C, Willems R: Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughters and (sub) urban residents in the south of The Netherlands: Evidence for transmission of vancomycin resistance in animals to humans? Antimicrob. Agents Chemotherp. (1999) 43:2215-2221
18. Ieven M, Vercauteren E, Descheemaeker P, van Laer F, Goossens H: Comparison of direct plating and broth enrichment culture for the detection of intestinal colonization by glycopeptide-resistant enterococci among hospitalized patients. J. Clin. Microbiol. (1999) 37:1436-1440.
19. Linden PK, Miller CB: Vancomycin-resistant enterococci: the clinical effect of a nosocomial pathogen. Dign. Microbiol. Infect. Dis. (1999) 33:113-20.
20. Ostrowsky BE, Venkataraman L, D'agata EM, Gold HS, DeGirolami PC, Samore HM: Vancomycin resistant enterococci in intensive care units: high frequency of stool carriage during a non-outbreak period. Arch. Intern Med. (1999) 159:1467-1472.
21. Dan M, Poch F, Leibson L, Smetana S, Priel I: rectal colonization with vancomycin resistant enterococci among high-risk patients in an Israeli hospital. J. Hosp. Infect. (1999) 43:231-238.
22. Falk PS, Winnike J, Woodmansee C, Desai M, Mayhall CG: Outbreak of vancomycin resistant enterococci in a burn unit. Infect. Control Hosp. Epidemiol. (2000) 21:575-582.
23. Wendt C, Krause C, Xander LU, Loffler D, Floss H: Prevalence and colonization with vancomycin-resistant enterococci in various population groups in Berlin. J. Hosp. Infect. (1999) 42:193-200.
24. Beltrami EM, Singer DA, Fish L, Manning K, Young S, Banerjee SN, Baker R, Jarvis WR: Risk factors for acquisition of vancomycin-resistant enterococci among patients on a renal ward during a community outbreak. Am. J. Infect. Control. (2000) 28:282-285.
25. Silverblatt FJ, Tibert C, Mikolich D, Blazek-D'arezzo J, Alves J, Track M, Agatiello P: Preventing the spread of vancomycin-resistant enterococci in a long-term facility. J. Am. Geriatr. Soc. (2000) 48:1211-1215.
26. Bates J: Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. J. Hosp. Infect. (1998) 37:89-101.
27. Pensack JM, Wang GT, Simkins KL: Avoparcin, a growth promoter promoting feed antibiotic for broiler chicken. Poultry Science (1982) 61:1009-1012.
28. Leeson S, Summers JD, Ferguson AE: Efficiacy of avoparcin as a growth promoter for broiler chicken. Can. J. Animal Scien. (1980) 60:275-279.
29. Aarestrup FM: occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from convential and ecological poultry farms. Microb. Drug. Resist. (1995) 3:255-257.
30. Robredo B, Singh KV, Baquerro F, Murray BE, Torres C: From *vanA Enterococcus hirae* to *vanA Enterococcus faecium*: a study of feed supplementation with avoparcin and tylosin in young chicken. Antimicrob. Agents Cheomotherp. (1999) 43:1137-1143.
31. Witte W: Selective pressure by antibiotic use in livestock. Int. J. Antimicrob. Agents. (2000) 16:S19-S24.

32. Mackinson C, Angulo F, Daugherty S, Johnson JA, Enterococci Resistance Study Team: Isolation of Vanocmycin-resitant *Enterococcus faecium* from commercial poultry. 40th ICAAC Toronto (2000) Abstract 1787.
33. Moller-Aarestrup F: Association between decreased susceptibility to a new antibiotic for treatment of human disease, everninomycin, and resistance to an antibiotic used for growth promotion in animals, avilamycin. Microb. Drug Resist. (1998) 4:137-141.
34. Van den Bogaard AE, Bruinsma N, Stobberingh EE: The effect of banning avoparcin on VRE carriage in The Netherlands. J. Antimicrob. Chemotherp. (2000) 46:146-148.
35. Schouten MA, Hoogkamp-Korstanje JAA, Klare I, Voss A: Gastro-intestinal tract colonization with vancomycin-resistant enterococci is associated with meat consumption. Veg. Nutrition (1998) 2:61-66.
36. Blom M, Sorensen TL, poulsen RL, Frimodt-Moller N, Monnet DL, Aarestrup FM, Espersen F: Ingestion of vancomycin-resistant *Enterococcus faecium* strains of food animal origin by human healthy volunteers: a randomized double-blind study. 40th ICAAC Toronto (2000) Abstract 1962.
37. Turabelidze D, Kotetishvili M, Kreger A, Morris jr JG, Sulakvelidze A: Improved Pulsed-Field Gel Electrophoresis for typing vancomycin-resistant enterococci. J. Clin. Microbiol. (2000) 38:4242-4245.
38. Vos P, Hogers R, Bleeker M, et al: AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. (1995) 23:4407–14.
39. Bootsma HJ, van der Heide HGJ, van de Pas S, Schouls LM, Mooi FR: Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. J Infect. Dis. (2000) 181:1376–1387.
40. Arnold C, Metherell L, Willshaw G, Maggs A, Stanley J: Predictive fluorescent amplified fragment length polymorphism analysis of *Escherichia coli:* high-resolution typing method with phylogenetic significance. J Clin. Microbiol. (1999) 37:1274–1279.
41. Duim B, Wassenaar TM, Rigter A, Wagenaar J. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. (1999) 65:2369–2375.
42. Grady R, Desai M, O'Neill G, Cookson B, Stanley J. Genotyping of epidemic methicillin-resistant *Staphylococcus aureus* phage type 15 isolates by fluorescent amplified fragment length polymorphism analysis. J Clin. Microbiol. (1999) 37:3198–3203.
43. Devriese LA, Cruz Colque JI, De Herdt P, Haesebrouck F. Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. J. Appl. Bacteriol. (1992) 73:421–425.
44. Devriese LA, Van de Kerckhove A, Kilpper-Balz R, Schleifer KH. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Sys. Bacteriol. (1987) 37:257–259.
45. Handwerger S, Skoble J, Discotto LF, Pucci M: Hetero-geneity of the *vanA* gene cluster in clinical isolates of enterococci from the Northeastern United States. Antimicrobial Agents Chemother. (1995) 39:362-368.
46. Woodford N, Adebiyi AA, Palepou MI, Cookson BD: Diversity of *vanA* glycopeptide resistance elements in enterococci from humans and non-human sources. Antimicrob. Agents Chemother. (1998) 42:502-508.
47. Jensen LB, Ahrens P, Dons L, Jones RN, Hammerum AM, Aarestrup FM: Molecular analysis of Tn1546 in *Enterococcus faecium* in isolates from animals and humans. J. Clin. Microbiol. (1998) 36:437-442.
48. Morrison D, Woodford N, Cookson BD: Epidemic vancomycin-resistant *Enterococcus faecium* in the UK. Clin. Microbiol. Infect. (1995) 1:146-147.
49. Pegues DA, Pegues CF, Hibberd PL, Ford DS, Hooper DC: Emergence and dissemination of a highly vancomycin-resistant *VanA* strain of *Enterococcus faecium* at a large teaching hospital. J. Clin. Microbiol. (1997) 35:1565-1570.
50. Bonten MJM, Hayden MK, Nathan C, Rice TW, Weinstein RA: Stability of vancomycin-resistant enterococcal genotypes isolated from long term colonized patients. J. Infect. Dis. (1998) 177:378-382.

51. Vandenbroucke-Grauls CMJE, Meesters HHM, Donkers LEA, Zaayer HL, Timmer GJ, Huijgens PC et al: Succesvolle bestrijding van een epidemie met vancomycine-resistente *Enterococcis faecium* op een hematologische afdeling. Ned. Tijdschr. Med. Microbiol. (2000) 8:S28.
52. Mascine EM, Gigengack-Baars ACM, Hene RJ, Kamp-Hopmans TEM, Weersink AJL, Bonten MJM: Epidemische verheffing van verscheidene genotypen van vancomycine-resistente enterococcen in een academisch ziekenhuis. Ned. Tijdschr. Geneeskd (2000) 144:2572-2576.
53. Van der Steen LF, Bonten MJM, van Kregten E, Harssema-Poot JJC, Willems R, Gaillard CA: Uitbraak van vancomycine-resistente *Enterococcus faecium* op een afdeling nefrologie. Ned. Tijdschr. Geneeskd (2000) 144:2568-2572.
54. Willems RJL, Homan W, Top J, van Santen-Verheuvel M, Tribe D, Manzioros X, Gaillard C, Vandenbroucke-Grauls CMJE, Mascini EM, van Kregten E, van Embden JDA, Bonten MJM. Variant esp gene as marker of a distinct genetic lineage of vancomycin resistant enterococcus faecium spreading in hospitals. Lancet (2001) 357:853-855.

Summary and Samenvatting

Enterococci are part of the normal human and animal gut-flora and hardly cause infections in healthy individuals. The last decade several enterococcal species have emerged as common causes of hospital-acquired infections. One of the major reasons why these microorganisms easily survive in the hospital environment is their intrinsic resistance to several commonly used antibiotics, and more importantly, their ability to acquire resistance to many currently used antibiotics, including the glycopeptides. Resistance development of enterocci to the glycopeptide vancomycin presents a major and worldwide noticed problem. Infections with vancomycin resistant enterococci are not only difficult to treat but the organisms show a strong propensity to disseminate and spread from patient to patient in the hospital setting. Glycopeptide-resistant enterococci can be divided in different classes depending on the presence of *van*-resistance genes. Resistance types can either be intrinsic (low-level resistance to vancomycin and teicoplanin; e.g. VanC1, VanC2 and VanC3) or acquired (high-level resistance to both vancomycin and teicoplanin [VanA], intermediate- level resistance to both glycopeptides [VanD, VanE] or variable level of resistance to vancomycin only [VanB]). The VanA/VanB resistances are encoded by homologous transposons named *Tn1546* and *Tn1547*, respectively. These transposons are located on self-transferable plasmids and can be transferred by conjugation to other bacteria. This thesis describes the detection, prevalence and molecular analysis of glycopeptide resistant enterococci in The Netherlands.

Detection of vancomycin resistance

Accurate and rapid detection of vancomycin resistance is essential in any strategy that aims to prevent nosocomial transmission of resistant organisms. In ***chapter 2***, we tested the accuracy of eight different susceptibility test methods for the detection of glycopeptide resistance in enterococci. *VanA* VRE strains were adequately detected by all methods. Despite the technical improvement of all new automated susceptibility test methods, E-test and the agar screen appeared to be the most reliable and easy-to-perform methods for routine screening of detection of *vanB*- and *vanC1/C2*-mediated resistance in enterococci. In ***chapter 3***, we tested a new fully automated susceptibility test method. The VITEK®2 approach presents an improvement over conventional methods for the detection of vancomycin resistance in enterococci. However, the

detection of teicoplanin resistance in enterococci containing the *vanA* gene needs to be reassessed. VITEK®2 is the first automated susceptibility method that tests for vancomycin- as well as teicoplanin- resistance, which is important for the description of the resistance phenotype. Although the detection time was reduced, further improvement of the algorithm and further reduction of the detection time may considerably increase the impact of rapid testing on patient care.

Prevalence of VRE in- and outside the hospital

Differences in epidemiology of VRE in the USA and Europe have been outlined before. In the USA the prevalence of VRE in the hospital setting increased enormously nationwide; many nosocomial VRE outbreaks have been described, and VRE were seldom found outside the hospital environment. In contrast, in Europe the VRE prevalence in hospitals remains low, VRE related outbreaks are rare although the isolation of VRE outside the hospital is common. It has been suggested that the frequent use of glycopeptides in hospitals in the USA and veterinary consumption of large amounts of glycopeptide-containing animal feeds in some countries of Europe have contributed to this scenario. Further gathering of epidemiological data will be useful to prevent the further spread of VRE. Therefore, an important aim of this thesis was to survey the prevalence of VRE colonization in and outside the hospital setting in The Netherlands and to gain more insight in the genetic relationship between these resistant bacteria.

In ***chapters 4 and 5***, the prevalence and determinants of VRE carriage in intensive-care units (ICU) and Hematology Oncology wards in nine Dutch hospitals and a non-hospitalized population (1995-1998) was determined. The prevalence of VRE colonization in The Netherlands remains low, as was determined for the rest of Europe by others. We have shown in a multicenter study that VRE can be isolated in hospitalized and in community-based patients in The Netherlands, at a frequency of 1.4% and 2%, respectively (chapter 4 and 5). Molecular analysis showed that all *E. faecalis* strains, harboring the *vanB* gene, were identical; these strains were isolated in a single ICU. After the end of the study period two additional patients from the same ICU were colonized with this VRE clone. This appeared to be the first nosocomial, clonal outbreak of colonization caused by VRE in The Netherlands. In contrast, when analyzing *E.*

faecium we found 4/11 genetically related strains but no geographical relationship. We reported that VRE carriage is not associated with prolonged hospital stay in a low endemicity country such as The Netherlands.

In a separate case-control study (***chapter 6a***) where the prevalence of VRE carriership between vegetarians versus meat eaters was analyzed, no significant association was found between the consumption of meat and high-level glycopeptide resistant bacteria in the gastro-intestinal flora. Remarkably, vegetarians are often carriers of *vanC* enterococci. We hypothesize that the consumption of plant products that are contaminated with mobile *E. casseliflavus* bacteria may be the source of the high prevalence of *vanC*-enterococci in vegetarians.

Chapter 6b described the prevalence of VRE in cats and dogs in Rotterdam, The Netherlands (chapter 6b). Although our data are representative of a single region only, we propose that domestic pets may be a significant reservoir for VRE. One of the VRE genotypes shared among dogs and cats was also found in a human carrier.

In ***chapter 7***, we reported an extremely high prevalence (79%) of VRE in poultry products. Total genome and transposon analysis show that transmission of the resistance genes, rather than clonal dissemination of resistant micro-organisms, may be the factor driving the spread of vancomycin resistance from poultry to humans.

Genome analysis of VRE

In the last three chapters several molecular techniques are described that can be applied to gain more insight into the spread of vancomycin resistant enterococci. The techniques we used were Pulsed Field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD) analysis, transposon analysis using PCR, Restriction Fragment Length Polymorphism (RFLP) analysis and sequencing, and Amplified Fragment Length Polymorphism (AFLP) analysis.

Chapter 8 showed that PFGE and RAPD analysis performed on diverse strains of VRE are largely concordant in outcome. This indicates that both methods can be used for adequate molecular typing. However, in case of clonal outbreaks we suggest to confirm the strain relatedness with the PFGE method, due to the higher discriminatory power of PFGE technique.

Chapter 9 reported seven different T*n1546* types using RFLP analysis of the VanA transposon of 97 VRE strains isolated from human and animal sources. Subsequent sequencing of a subset of these RFLP types resulted in the identification of 22 different T*n1546* derivatives. Identical T*n1546* types were found among isolates from humans and farm animals in The Netherlands, suggesting the sharing of common vancomycin resistance gene pools. Application of the genetic analysis of T*n1546* to VRE isolates causing infections in hospitals in the United Kingdom and the USA suggested that horizontal transmission of the vancomycin resistance transposon might occur in a clinical setting.

Finally, ***chapter 10*** displayed that VREF strains are predominantly host-specific, and strains isolated from hospitalized patients are genetically different from the prevailing VREF strains present in the fecal flora of nonhospitalized persons. AFLP and PFGE analysis were used to investigate the genetic relationships among 255 vancomycin-resistant *Enterococcus faecium* (VREF) strains isolated from hospitalized patients, nonhospitalized persons, and various animal sources. Four major AFLP genogroups were discriminated; group A: non-hospital isolates and pig isolates, group B: poultry and turkey isolates, group C: hospital isolates, calve isolates and dog/cat isolates, and group D: calve isolates. Molecular typing of T*n1546* in the VREF strains analyzed in this study shows the various VanA transposon variants are not randomly distributed among the four main VREF genogroups identified. Some transposon types are predominantly found in only one genogroup, thus exhibiting a high degree of host specificity. Other transposon types seem to be more promiscuous, as these are found in most VREF genotypes.

In conclusion, the main findings presented in this thesis are that VRE are still rare among hospitalized patients in The Netherlands. However, high carriage rates of VRE can be documented in the open population and in chicken meat products. Population genetics of VRE revealed restricted host specificity, which may in part explain the low prevalence in hospitals in a typical clinical setting where antibiotic use is restricted.

Enterococcen zijn bacteriën die behoren tot de normale darmflora van mens en dier en veroorzaken zelden infecties bij gezonde individuen. Echter, de laatste tientallen jaren ontwikkelden deze bacteriën zich tot een belangrijke groep van veroorzakers van ziekenhuisinfecties. Eén van de redenen waarom deze micro-organismen kunnen overleven in het ziekenhuis is hun aangeboren resistentie tegen een aantal veel gebruikte antibiotica en, nog belangrijker, hun vermogen om additionele resistenties te verwerven tegen de meeste antibiotica, inclusief de glycopeptiden. Resistentie ontwikkeling van enterococcen tegen het glycopeptide vancomycine is een groot en wereldwijd probleem. Infecties met vancomycine resistente enterococcen zijn moeilijk te behandelen en deze organismen vertonen de neiging om zich te verspreiden in een ziekenhuis setting, bijvoorbeeld van patiënt tot patiënt. Glycopeptide resistente enterococcen kunnen verdeeld worden in verschillende klassen afhankelijk van het *van*-resistentie gen dat in de bacterie aanwezig is. Resistentie typen kunnen "aangeboren" zijn (lage resistentie tegen de glycopeptiden vancomycine en teicoplanine; VanC1, VanC2 en VanC3) of verkregen (hoge resistentie tegen vancomycine en teicoplanine [VanA], intermediaire resistentie tegen beide glycopeptide [VanD, VanE] of variabele resistentie tegen vancomycine alleen [VanB]). De VanA en VanB resistenties worden gecodeerd door transposons (kleine mobiele stukjes DNA die in het genoom van plaats kunnen wisselen en mede verantwoordelijk zijn voor de regeling van de resistentie gen-activiteit) zoals Tn1546 en Tn1547. Deze transposons liggen vaak op plasmiden (circulair DNA) die kunnen worden overgedragen naar andere bacteriën.

Dit proefschrift beschrijft de detectie, prevalentie en moleculaire analyse van glycopeptide-resistente enterococcen in Nederland.

Detectie vancomycine resistentie

Accurate en snelle detectie van vancomycine resistentie is essentieel in elke strategie die als doel heeft om te voorkomen dat verspreiding van deze resistente micro-organismen binnen het ziekenhuis plaats vindt. In ***hoofdstuk 2***, wordt de nauwkeurigheid van 8 verschillende methodes voor de detectie van glycopeptide-resistentie in enterococcen getest. We laten zien dat *VanA* gemedieerde vancomycine resistentie goed wordt gedetecteerd door alle methodes. Ondanks de beschikbaarheid en de verbetering van (nieuwe)

automatische resistentie test methodes, blijken E-test en vancomycine screen agar de meest betrouwbare en makkelijk uit te voeren methodes te zijn voor het routinematig screenen van *vanB* en *vanC1/C2* resistentie in enterococcen. In *hoofdstuk 3* wordt een nieuw volautomatisch resistentie test systeem getest. Vitek®2 levert een duidelijke verbetering ten opzichte van andere automatische methodes. Echter, de detectie van teicoplanine resistentie in enterococcen die het *vanA* gen bevatten vergt echter nog enige optimalisering. VITEK®2 is het eerste vol automatische test systeem dat zowel vancomycine als teicoplanine resistentie test, wat belangrijk is voor de benaming van het resistentie fenotype bij glycopeptide resistentie. Verdere reductie van de detectie tijd zal een grote impact hebben op de patiëntenzorg.

Prevalentie van VRE binnen en buiten het ziekenhuis

Er is bekend dat er verschillen bestaan tussen Amerikaans en Europese epidemiologie van VRE. In de VS is de VRE prevalentie in ziekenhuizen enorm toegenomen, vele VRE uitbraken op afdelingen zijn beschreven, maar een VRE wordt zelden buiten de ziekenhuissetting geïsoleerd. In Europa daarentegen is de VRE prevalentie binnen de ziekenhuizen laag, zijn er nauwelijks uitbraken beschreven, maar er is wel bekend dat er buiten de ziekenhuisomgeving ook VRE geïsoleerd worden. Gesuggereerd wordt dat het hoge gebruik van glycopeptiden in Amerikaanse ziekenhuizen en van glycopeptide-bevattend dierenvoedsel in sommige landen in Europa heeft bijgedragen aan deze sterk verschillende scenario's. Verdere uitbreiding van epidemiologische surveillances zijn van belang om verdere verspreiding van VRE te voorkomen. Een belangrijk doel van dit proefschrift is het bepalen van de prevalentie van VRE in en buiten het ziekenhuis in Nederland en meer inzicht te verkrijgen in de genetische relatie tussen deze resistente bacteriën.

In ***hoofdstukken 4 en 5***, worden de prevalentie en enkele risicofactoren van VRE dragerschap op intensive-care-(IC) en hematologie-oncologie afdelingen in negen Nederlandse ziekenhuizen en een niet ziekenhuis populatie tussen 1995 en 1998 onderzocht. De prevalentie van VRE kolonisatie in Nederland blijft laag, net als in de rest van Europa, in tegenstelling tot de situatie in Amerika. We hebben aangetoond dat VRE dragerschap voorkomt in ziekenhuis- en in de gewone populatie met een frequentie van 1.4% en 2%. Moleculaire analyse van

ziekenhuis-VRE laat zien dat alle gedetecteerde *Enterococcus faecalis* isolaten (n=3) het *vanB* gen hebben en genetisch identiek zijn. Deze stammen werden geïsoleerd op één IC afdeling. Na afloop van de studieperiode zijn twee additionele patiënten van dezelfde IC afdeling gekoloniseerd met deze VRE kloon. Dit is de eerste ziekenhuis gerelateerde VRE-"uitbraak" in Nederland. Analyse van *Enterococcus faecium* (*vanA*) isolaten laat zien dat 4 van de 11 isolaten genetisch maar niet geografisch gerelateerd zijn. Na het onderzoeken van verschillende risicofactoren zien we, in tegenstelling tot een aantal andere studies, dat VRE dragerschap in Nederland niet geassocieerd is met langdurig verblijf in het ziekenhuis.

In een case-control studie (***hoofdstuk 6a***) waarin prevalentie van VRE dragerschap in vegetariërs versus vleeseters wordt geanalyseerd, wordt geen significante associatie gevonden tussen de consumptie van vlees en VRE dragerschap bij mensen. Opvallend is wel dat vegetariërs beduidend vaker drager zijn van *vanC* enterococcen. We suggereren dat de consumptie van plantaardige producten, wat weer geassocieerd kan worden met het eten van plantaardige producten die gecontamineerd kunnen zijn met de beweeglijke *E. casseliflavus* bacterie, de bron zou kunnen zijn van de hoge prevalentie *vanC* enterococcen in vegetariërs.

Hoofdstuk 6b beschrijft de VRE prevalentie bij katten en honden in Rotterdam, Nederland. Onze data zijn representatief voor slechts één regio, maar toch is het duidelijk dat huisdieren een significant VRE reservoir kunnen vormen. Eén van de VRE genotypen gevonden bij zowel honden als katten is ook gevonden bij een menselijke drager.

In ***hoofdstuk 7*** rapporteren we een extreem hoge VRE prevalentie (79%) in kipproducten. Totaal genoom- en transposon- analyse laten zien dat transmissie van de resistentie genen, eerder dan klonale verbreiding van resistente micro-organismen, een factor kan zijn voor de verspreiding van vancomycine resistentie van kip naar mens.

Genoom analyse van VRE

In de laatste drie hoofdstukken worden verscheidene moleculaire technieken beschreven die gebruikt kunnen worden om meer inzicht te krijgen in de verspreiding van vancomycine resistente enterococcen. De technieken die

gebruikt worden zijn Pulsed Field gel Electroforese (PFGE), Random Amplificatie van Polymorf DNA (RAPD), transposon analyse door gebruik van Polymerase Chain Reactie gevold door Restrictie Fragment Lengte Polymorfisme (RFLP) en sequencen, en Amplified Fragment Lengte Polymorfisme (AFLP).

Hoofdstuk 8 laat zien dat de PFGE en RAPD analyses uitgevoerd op verschillende type VRE stammen overeenkomstige uitslagen genereren. Dit duidt erop dat beide technieken bruikbaar zijn voor moleculair typeren van deze bacteriën. Echter, als er sprake is van een klonale VRE uitbraak aan de hand van RAPD analyse wordt geadviseerd dit te bevestigen met PFGE analyse omdat de PFGE techniek een hoger discriminerend vermogen heeft.

Hoofdstuk 9 beschrijft 7 verschillende *Tn1546* transposon types, die geïdentificeerd werden na RFLP analyse van 97 VRE isolaten afkomstig van mens en dier. Sequencen van deze verschillende RFLP types resulteerde in 22 verschillende *Tn1546*-derivaten. Identieke *Tn1546* types werden gevonden bij isolaten afkomstig van mens en boerderij dieren, dit suggereerde disseminatie van gelijke vancomycine resistentie genen. Het toepassen van deze genetische analyse van de Tn1546 transposon op infectie veroorzakende VRE isolaten in een ziekenhuis in Engeland en Amerika laat horizontale transmissie zien van het vancomycine resistentie transposon.

Tenslotte, ***hoofdstuk 10*** laat zien dat vancomycine resistente *Enterococcus faecium* (VREF) isolaten overwegend gastheerspecifiek zijn, en isolaten van ziekenhuis patiënten genetisch verschillen van VREF isolaten die aanwezig zijn in de fecale flora van niet-gehospitaliseerde personen. AFLP analyse is gebruikt om de genetische relatie tussen 255 VREF stammen geïsoleerd van ziekenhuis patiënten, niet-gehospitaliseerde personen en verschillende dieren vast te stellen. Vier grote AFLP groepen worden onderscheiden; groep A: niet ziekenhuis isolaten en varkens isolaten, groep B: kip-en kalkoen isolaten, groep C ziekenhuis isolaten, kalf isolaten en hond/kat isolaten, en groep D: kalf isolaten. Moleculaire analyse van het Tn1546 transposon in de VREF isolaten laat zien dat het aantal VanA transposon varianten niet random verdeeld is onder de 4 AFLP groepen. Sommige transposon types komen voornamelijk voor in 1 van de groepen en suggereren hiermee een hoge gastheer specificiteit te hebben. Andere transposon types zijn meer verspreid en zijn te vinden in de meeste geno-groepen.

In conclusie, de belangrijkste bevindingen in dit proefschrift zijn dat VRE zeldzaam voorkomen bij ziekenhuis patiënten in Nederland. Echter, significant dragerschap van VRE wordt wel gerapporteerd in de open populatie en in de meeste kippenvlees producten. Populatie genetica van VRE laat een beperkte mate van gastheer specificiteit zien, wat voor een deel een verklaring kan zijn voor de lage prevalentie in ziekenhuizen in een klinische situatie waar het antibiotica gebruik beperkt is.

Dankwoord

En dan tot slot het dankwoord, het hoofdstuk wat als eerst zorgvuldig onder de loep genomen wordt door vele lezers. En terecht want zo'n boekje schrijven doe je tenslotte niet in je eentje. Dus wil ik graag iedereen bedanken die op welke manier dan ook een bijdrage heeft gehad aan het tot stand komen van dit proefschrift. Maar om het niet al te onpersoonlijk te maken zijn er een heleboel mensen die ik nog even wil noemen.

Als eerste de twee, voor mij belangrijkste, mensen die in dit geheel betrokken zijn, mijn copromotoren: **Hubert Endtz** en **Alex van Belkum**. Bedankt voor jullie enorme inzet om van een eenvoudige HLO student als ik een wetenschappelijk (ahum) onderzoekster te maken. Hubert, graag zou ik je in het Frans merci-en maar aangezien mijn Franstalige woordenkennis niet verder reikt dan du pain, du vin et du boursin toch maar in het Nederlands. Zonder jou enthousiasme over alle bacteriën uit het darmkanaal en me uit de routine weg te kapen had dit proefschrift hier nooit gelegen. Alex, ik weet dat je hier absoluut niet van houdt maar het diep in je hart wel fijn vindt. Bedankt voor het wegwijs maken in de wereld van de moleculaire biologie en voor het tig keer doorlezen en hartelijk lachen om de teksten van mijn artikels en presentaties.

Mijn promotor, **Henri Verbrugh**. Beste Henri, bedankt dat je me de kans gegeven hebt om me te bewijzen als onderzoekster en voor je begeleiding bij het in elkaar steken van dit boekje. We zijn ongeveer in dezelfde tijd op de afdeling gekomen, jij als prof ik als stagiaire, ik kan me nog goed herinneren toen ik werd aangenomen je naar me toe kwam en zei: Vertel mij is hoe jij je toekomst hier ziet? Hoofdanalist riep ik enthousiast, maar ik moet zeggen deze weg bevalt me een stuk beter.

Marian Humphrey, bedankt voor de engelse correctie van de voor jou meestal hocus pocus verhalen die ik op papier zet. Ik heb in die jaren veel opgestoken van je uitleg over de engelse grammatica.

Rooie, paarse, roze **Cindy** en **Deb**, we worden toch al als een eenheid gezien dus ik kan jullie best in een aparte alinea zetten. Ik wil even kwijt dat ik het geweldig vind om jullie zowel op het werk als buiten het werk

om me heen te hebben en voor jullie begrip dat ik andere paranimfen heb gekozen. Danki!!

En natuurlijk, **Suus**, dunne **Nicole** en **Hélène**. Bedankt voor de gezellige samenwerking tijdens mijn onderzoek en ik beloof dat er nooit meer vieze luchten in het lab hangen.

Willem, ouwe rot in het vak, bedankt voor de gezellige tijd in het buitenland. Ik vond het allemaal best wel spannend om de eerste keer met mijn poster op de ICAAC rond te lopen, maar jij hebt laten zien dat het allemaal heel relaxed is. Ik hoop dat we in de toekomst nog vaak samen op pad gaan en veel margaritas mogen drinken. Succes met je promotie!

Stagiaires, onmisbaar in het onderzoeksleven. Bedankt voor de bergen werk die jullie verzet hebben, het leren begeleiden en alle gezelligheid. **Marrit**, voor het mee verzamelen en typeren van al die kippen enterococcen. **Deborah**, goed dat je al bij de PTT werkte want hoe hadden we anders die duizend pakketjes moeten versturen. **Reneetje**, heb je nog nachtmerries over MIC bepalingen? Sorry! **Liset**, stip..stip..stip..stip wel of geen eiwit en we hebben er eigenlijk nooit iets mee gedaan, maar het is nog niet te laat. **Jamal**, al die kipfilets die je onderzocht heb op vancomycine resistente *S. aureus*, jammer dat we niets gevonden hebben maar het leven van een onderzoeker gaat niet altijd over rozen. **Remke, Debbie** en **Diana**, geen enterococcen maar campylobacters maar wat maakt het uit ze komen allemaal uit hetzelfde kanaal, bedankt!

Hennie, Loes, Marja, Patricia, Marieke, Klaartje, Kimberly, Hélène en **Carla**, bedankt voor het 3x daagse rook plezier buiten in alle weersomstandigheden. Zonder jullie was stelling 10 er nooit geweest.

Te veel om iedereen persoonlijk te noemen, maar ook alle **collega's van het baclab, ziekenhuis hygiënische dienst, secretariaat en de 17de verdieping**, bedankt voor alle gezelligheid op de werkvloer en de vele etentjes en labdagen.

Wendy, bedankt voor alle gezellige carpool jaren, ondertussen kennen we elke sluiproute en staan we niet meer raar te kijken van rare objecten op de weg.....staat daar nou een koe op de weg?

Rob Willems en **Janetta Top**, de laatste twee hoofdstukken maken dit proefschrift compleet. Bedankt dat ik ze mocht gebruiken voor de finishing touch en voor de samenwerking.

Eddy Power and **Richard Anthony**, thanks for the co-operation. Due to the distance between our labs it wasn't always easy to communicate, but a lot of e-mails, faxes and telephone calls resulted in an article and chapter 8 of this thesis.

De **Werkgroep ZIEN** (**Jan, Christina, Annemarie, Lodewijk, Hans, Andreas, Anton**) bedankt voor de jaarlijkse VRE screeningen, zonder jullie enthousiasme en inzet was het me nooit gelukt een begin te maken aan dit onderzoek.

Hans van Boven, directeur Nederlandse Bond voor Vegetariërs, en alle **vegetariërs** die meegewerkt hebben aan het onderzoek. Achteraf denk je is het niet raar om mensen per post wattenstokken op te sturen, met als vraag bij zichzelf en een bekend vleesetend medemens een perineum uitstrijk te maken. Maar dat het niet raar was bleek wel uit de enorme medewerking van jullie kant, bedankt.

Keurings Dienst van Waren Nederland, bedankt voor jullie enorme inzet en met name **John Vliegenthart** voor de assistentie bij het opzetten van het onderzoek.

Ronald Thomassen, via Els hebben we je weten te strikken om in je praktijk honden en katten te screenen, anders had ik zelf met wattenstokken achter huisdieren aan moeten rennen.

Martijn La Feber, bedankt voor het mooie kaffie!! Ik zal even reclame voor je maken. LFBR, voor al u internet design!

Diny, bedankt voor de koppies thee als ik op vrijdag thuis werkte om dit proefschrift in elkaar te zetten.

Mijn paranimfen, **Carla** en **Nancy**. In hoeverre ik jullie als paranimf moet bedanken weet ik nog niet, de verdediging van proefschrift moet tenslotte nog komen, maar ik heb er alle vertrouwen in dat alles goed gaat als jullie maar naast me staan! Thanks for being a friend.

Pap en mam, het is heel simpel zonder jullie had ik niet bestaan en was dit proefschrift er niet geweest. Bedankt voor alles.

En dan natuurlijk niet te vergeten **BAS**, speciaal voor jou een aparte pagina, mooier kan het niet uit komen, toch? Door jou is mijn HLO tijd geweldig geweest, en die jaren erna nog veel beter. Sorry dat ik je eetlust soms heb bedorven als ik weer vrolijk begon te vertellen over bacteriën, poep en enge ziektes.

Zo dat wilde ik even kwijt!

List of Publications

Pittuch H, **van den Braak N**, van Leeuwen WB, van Belkum A, Martirosian G, Obuch-Woszcatynski P, Luczack M and Meisle-Mikolajczyk. Clonal dissemination of a toxin-A negative/toxin-B positive *Clostridium difficile* strain from patients with antibiotic associated diarrhoea in Poland. In press **Clin Microbiol and Infect**

Dingle KE, **van den Braak N**, Colles FM, Endtz H. Ph, van Belkum A, Maiden MCJ. Sequence Typing confirms that *Campylobacter jejuni* strains associated with Guillain-Barre and Miller-Fisher Syndromes are of a diverse genetic lineage, serotype and flagella type. **J of Clin Microbiol**, 2001; Sep 39(9): 3346-9

van Belkum A, **van den Braak N**, Godschalk PCR, Ang CW, Jacobs B, Gilbert M, Wakarchuk W, Verbrugh HA and Endtz HP. A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. **Nature of Medicine**, 2001; Jul (7): 752-753.

Livermore DM, Carter MW, Bagel S, Wiedemann B, Baquero F, Loza E, Endtz HP, **van den Braak N**, Fernandes CJ, Fernandes L, Frimodt-Moller N, Rasmussen LS, Giamarellou H, Giamarellos-Bourboulis E, Jarlier V, Nguyen J, Nord CE, Struelens MJ, Nonhoff C, Turnidge J, Bell J, Zbinden R, Pfister S, Mixson L, Shungu DL. In Vitro Activities of Ertapenem (MK-0826) against Recent Clinical Bacteria Collected in Europe and Australia. **Antimicrob Agents Chemother** 2001 Jun; 45(6): 1860-7

Van den Braak N, van Belkum A, Kreft D, Verbrugh H, Endtz H. Dietary habits and gastrointestinal colonization by antibiotic resistant microorganisms. **J Antimicrob Chemother** 2001 Apr; 47(4): 498-500

Van den Braak N, Goessens W, Van Belkum A, Verbrugh H, Endtz H. Accuracy of the VITEK2 system to detect vancomycin- and teicoplanin resistance in enterococci. **J Clin Microbiol**. 2001 Jan; 39(1): 351-3.

Van den Braak N, Power E, Endtz H, Verbrugh H, Van Belkum A. Random amplification of polymorphic DNA versus pulsed field gel electrophoresis of *SmaI* DNA -nacrorestriction fragments for strains of VRE. **FEMS Microbiol Lett**. 2000 Nov; 192(1): 45-52.

Endtz HP, Ang CW, **van den Braak N**, Luijendijk A, Jacobs BC, de Man P, van Duin JM, van Belkum A, Verbrugh HA. Evaluation of a new commercial immunoassay for rapid detection of Campylobacter jejuni in stool samples. **Eur J Clin Microbiol Infect Dis** 2000 Oct; 19(10): 794-7

Willems RJ, Top J, **van den Braak N**, van Belkum A, Endtz H, Mevius D, Stobberingh E, van Den Bogaard A, van Embden JD. Host specificity of vancomycin-resistant *Enterococcus faecium*. **J Infect Dis** 2000 Sep; 182(3): 816-23

Van den Braak N, Ott A, Van Belkum A, Kluytmans J, Koeleman J, Spanjaard L, Voss A, Weersink A, Vandenbroucke-Grauls C, Buiting A, Verbrugh H, Endtz P. Prevalence and determinants Of fecal colonization with VRE in hospitalized patients in The Netherlands. **Infect Control Hosp Epidemiol**. 2000 Aug; 21(8): 520-4.

Endtz H, Ang W, **Van den Braak N,** Duim S, Rigter A, Price L, Woodward D, Rodgers F, Johnson W, Wagenaar J, Jacobs B, Verbrugh H, Van Belkum A. Molecular characterisation of C. *jejuni* from patients with Guillain-Barre and Miller Fisher Syndrome. **J Clin Microbiol**. 2000 Jun; 38(6): 2297-301.

Van den Braak N, van Belkum A, Kreft D, te Witt R, Verbrugh HA, Endtz HP. The prevalence and clonal expansion of high-level gentamicin-resistant enterococci isolated from blood in a Dutch university hospital. **J Antimicrob Chemother**. 1999 Dec; 44(6): 795-8.

Endtz HP, **van den Braak N,** Verbrugh HA, van Belkum A. Vancornycin resistance: status quo and quo vadis. **Eur J Clin Microbiol Infect Dis**. 1999 Oct; 18(10): 683-90.

Willems RJ, Top J, **van den Braak N,** van Belkum A, Mevius DJ, Hendriks G, Vansanten-Verheuvel M, van Embden JD. Molecular diversity and evolutionary relationships of Tnl546-like elements in enterococci from humans and animals. **Antimicrob Agents Chemother**. 1999 Mar; 43(3): 483-91.

Van den Braak N, van Belkum A, te Witt R, Verbrugh HA, Endtz HP. Glycopeptide resistance amongst Staphylococcus spp. **J Antimicrob Chemother**. 1998 Nov; 42(5):673-5

van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HP. Molecular characterization of VRE from hospitalized patients and poultry products in The Netherlands. **J Clin Microbiol**. 1998 Jul; 36: 1927-32.

Endtz HP, **Van Den Braak N,** Van Beikum A, 'oessens WH, Kreft D, Stroebel AB, Verbrugh HA. Comparison of eight methods to letect VRE. **J Clin Microbiol**. 1998 Feb; 36(2): 592-4.

Endtz HP, **van den Braak N,** van Belkum A, Kluytmans JA, Koeleman G, Spanjaard, Voss A, Weersink AJ, Vandenbroucke-Grauls CM, Buiting AG, van Duin A, Verbrugh HA. Fecal carriage of VRE in hospitalized patients and those living in the community in The Netherlands. **J Clin Microbiol**. 1997 Dec; 35(12): 3026-31.

Van den Braak N, Kreft D, van Belkum A, Verbrugh H, Endtz H. VRE in vegetarians. **Lancet**. 1997 Jul 12; 350(9071): 146-7.

Mouton JW, Endtz HP, den Hollander JG, **van den Braak N,** Verbrugh HA. In-vitro activity of quinupristin/dalfopristin compared with other widely used antibiotics against strains isolated from patients with endocarditis. **J Antimicrob Chemother**. 1997 May; 39 Suppl A: 75-80.

Endtz HP, Mouton JW, den Hollander JG, **van den Braak N,** Verbrugh HA.Comparative in vitro activities of trovafloxacin (CP- 9,219) against 445 gram-positive isolates from patients with endocarditis and those fith other bloodstream infections. **Antimicrob Agents Chemother.** 1997 May; 41(5): 1146-9.

Van Belkum A, Vandenbergh A, Kessie G, Qadri SM, Lee G, **van den Braak N,** Verbrugh H, al-Ahdal MN. Genetic Homogeneity among MRSA strains from Saudi Arabia. **Microb Drug Resist.** 1997 winter 3: 365-9.

Endtz HP, Vliegenthart JS, Vandamme P, Weverink HW, **van den Braak N** , Verbrugh HA, van Belkum A. Genotypic diversity of C. lari isolated from mussels and oysters inthe Netherlands. **Int J Food Microbiol.** 1997 Jan ;34(l): 79-88.

van Belkum A, **van den Braak N,** Thomassen R, Verbrugh H, Endtz H. Vancomycin-resistant enterococci in cats and dogs. **Lancet.** 1996 Oct 2; 348(9033): 1038-9.

Curriculum Vitae

Nicole van den Braak werd op vrijdag 4 augustus 1972 in Tilburg geboren. Na haar middelbare school rond zij in 1995 met succes het Hoger Laboratorium Onderwijs af aan de Polytechnische Faculteit in Etten-Leur, met als specialisatie Medische Microbiologie. Sinds het afronden van deze opleiding is zij werkzaam op de afdeling Medische Microbiologie en Infectieziekten van het Erasmus Universitair Medisch Centrum Rotterdam (EMCR). Vanaf die tijd was zij voornamelijk betrokken bij wetenschappelijk onderzoek onder begeleiding van Dr. H. Ph. Endtz en Dr. A. van Belkum. In de loop der jaren heeft zij onderzoek verricht met name op het gebied van vancomycine resistentie in enterococcen wat resulteerde in dit proefschrift met als promotor Prof. Dr. H.A. Verbrugh. Gedurende dezelfde periode was zij ook betrokken in het onderzoek wat gericht is op de relatie tussen het Guillain-Barré Syndroom en *Campylobacter jejuni.* Dit onderzoek is uitgevoerd in samenwerking met de afdelingen Immunologie en Neurologie van dezelfde Universiteit, Institute for Biological Science, Ottowa, Canada en het Analytisch Diagnostisch Centrum in Curacao, Nederlandse Antillen waar zij in 1999 een maand lang heeft gewerkt. Na de promotie zal zij werkzaam blijven op de afdeling Medische Microbiologie en Infectieziekten van het EMCR.