SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

MOLECULAR CHARACTERIZATION OF VANCOMYCIN-RESISTANT ENTEROCOCCI

by Dorottya Frányó Kocsiné

Supervisor: Dr. Zsuzsanna Dombrádi, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES
DEBRECEN, 2019
Molecular characterization of vancomycin-resistant enterococci

By Dorottya Frányó Kocsiné

Supervisor: Dr. Zsuzsanna Dombrádi, PhD

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the Examination Committee: Prof. Dr. Árpád Tóssy, PhD, DSc

Members of the Examination Committee: Prof. Dr. Ferenc Rozgonyi, PhD, DSc
Prof. Dr. Sándor Biró, PhD, DSc

The Examination takes place at Library of Department of Pharmacology, Faculty of Medicine, University of Debrecen, 20th of January 2020 at 11:00

Head of the Defense Committee: Prof. Dr. Árpád Tóssy, PhD, DSc
Reviewers: Dr. Eszter Ostorházi, PhD
Dr. Anikó Ujfalusi, PhD

Members of the Defense Committee: Prof. Dr. Ferenc Rozgonyi, PhD, DSc
Prof. Dr. Sándor Biró, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 20th of January 2020 at 13:00
INTRODUCTION

Antimicrobial resistance is one of the most serious health threats. Infections caused by resistant bacteria are emerging, and some pathogens have even become resistant to multiple classes of antibiotics. The latest report of the Centers for Disease Control and Prevention (CDC) sounded the alarm to the dangers of antibiotic resistance, stating that each year in the U.S. at least 2 million people suffer from an antibiotic-resistant infection, and more than 23,000 people die.

Enterococci cause a range of diseases such as sepsis, wound and urinary tract infections, especially among patients receiving medical care. 30% of healthcare-associated enterococcal infections are vancomycin-resistant. The clinically relevant species are: Enterococcus faecalis, Enterococcus faecium, E. gallinarum and E. casseliflavus. According to the CDC, 77% of VRE infections are caused by Enterococcus faecium and 9% by Enterococcus faecalis. The latest data of the European Centre for Disease Prevention and Control (ECDC) showed that the prevalence of VRE E. faecium (VREfm) has been steadily increasing among Hungarian hospitalized patients. This report demonstrated a rise from 0.8% to 39.5% among invasive isolates in Hungary between 2011 and 2018.

Concerning the epidemiology of VRE, we know that the appearance and spread of these strains has a different background in Europe and in the United States. While in the USA the widespread usage of vancomycin and/or cephalosporin in human treatment, in Europe, the application of avoparcin as a growth-promoting factor in animal husbandry lead to the appearance of VRE. Enterococci are ubiquitous in gastrointestinal tracts even though they constitute a small proportion approx. 0.01% of the normal bowel flora. Typical concentrations in stool are up to 10^8 Colony Forming Unit (CFU)/gram in humans. A number of risk factors have been associated with VRE colonization. These include prolonged hospital stay, advanced age, severe underlying disease, central venous catheterization and exposure to various antibiotics such as vancomycin or metronidazole. Asymptomatic carriage of VRE and the lack of effective decolonization regimen stabilize their endemicity in the healthcare settings. This colonization may serve as a reservoir for the transmission to other patients resulting in increased morbidity, mortality and cost.

Under these circumstances the significance of resistance surveillance has risen. Monitoring the presence of resistant strains, and identifying potential reservoirs is essential for determining appropriate empirical antibiotic therapy. With my research work I wanted to contribute to this area by analyzing vancomycin-resistant enterococci at the Bacteriological Diagnostic Laboratory of the Department of Medical Microbiology.
AIMS

The goal of my work was the epidemiological and molecular characterization of VRE isolated from clinical samples and to determine the carriage rates of VRE from stool samples. We also investigated various risk factors which could be associated with VRE colonization.

Investigation of vancomycin-resistant enterococcus strains isolated from clinical specimens:

Aims:
1. Screening of VRE strains isolated from clinical samples collected between 2012-2015.
3. Antibiotic susceptibility testing, and molecular identification of antibiotic resistance and virulence genes.
4. Investigating the association between virulence factors and biofilm formation.
5. Investigating the epidemiological relationships between the isolates by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Investigation of vancomycin-resistant enterococcus strains isolated from stool:

Aims:
1. Screening of VRE strains isolated from stool samples collected between February and September 2016 by using two screening methods.
2. Identification of VRE by MALDI-TOF MS.
3. Antibiotic susceptibility testing and molecular identification of the resistance genes.
4. Characterization of VRE positive patients and carriers.
5. Analysis of risk factors for VRE colonization.
MATERIALS AND METHODS

Isolates
Clinical samples were collected between January 2012 and December 2015. Isolates were cultured on VRE screening plates containing 6 µg/mL vancomycin. The period of the stool sample examination was divided into two phases. In the 1st period (155 days, 2050 specimens) stools were inoculated with a glass stick onto solid VRE screening medium containing 6µg/mL vancomycin. In the 2nd period (88 days, 971 specimens) the same inoculation technique and culture medium was used but without solidification with agar (broth enrichment).

Antimicrobial susceptibility testing
All protocols were performed according to the manufacturer’s instructions. The European Committee on Antimicrobial Susceptibility Testing guidelines were used to interpret the results. Determination of antibiotic susceptibility patterns was performed by Kirby-Bauer disc diffusion using ampicillin (2µg), gentamicin (30µg) and ciprofloxacin (5µg) antibiotic discs. E-test is the most appropriate method for determining MIC values in the routine diagnosis. On the surface of the E-test strips there are well defined concentration gradients of antibiotics. After the appropriate incubation period an elliptical growth inhibition zone will develop. Vancomycin, teicoplanin, daptomycin, tedizolid, linezolid, tigecycline and quinupristin/dalfopristin minimum inhibitory concentrations (MICs) were determined by E-test.

Molecular biology techniques

Polimerase chain reaction
Templates of the PCR reactions were bacterial lysates prepared by the boiling method of Yean and colleagues (2007). PCR conditions and primers for the VanA, VanB, VanC, VanD, VanE, and VanG resistance genes as well as for E. faecalis and E. faecium D-alanine:D-alanine ligase (ddl) were taken from Depardieu et al (2004). The oligonucleotide primers used in virulence gene (agg, ace esp) detections were described previously by Woodford et al., Eaton et al., and Mannu et al. PCR reactions were performed according to Cariolato and colleagues (2008). Primers for the MLST experiments were used according to the https://pubmlst.org/ website. The results of the PCR reactions and were determined by agarose gel electrophoresis.

Testing biofilm formation
To study the biofilm forming ability of the VRE strains a modified microtiter-plate test was performed based on the protocol of Stepanovic et al. (2007) with some modifications. This method is superior to the standard test because it measures bacteria attached both to the bottom and walls of ELISA plates. The optical density (OD) was measured photometrically at 540/620nm. The optical density cut-off (ODc) was defined as the standard deviations above the mean OD of the negative control. Isolates were classified as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ 2 × ODc = weak biofilm producer, 2 × ODc < OD ≤ 4× ODc = moderate biofilm producer, 4 × ODc < OD= strong biofilm producer.
Methods of epidemiological typing

Pulsed-field Gel Electrophoresis (PFGE)

PFGE of VRE strains was performed according to the protocol of Murray et al. and McEllistrem et al. at the National Public Health Center, with slight modifications. PFGE-generated DNA profiles were entered into the Fingerprinting II version 3.0 Software using 1.0% position tolerance and 1.0% optimization setting. The quantitative differences among the banding patterns were defined by the Dice coefficient. Unweighted pair group method with arithmetic mean (UPGMA) was used for cluster analysis of the PFGE patterns.

Multilocus sequence typing (MLST)

Internal fragments of seven housekeeping genes (adk, atpA, ddl, gdh, gyd, purK and pstS) were amplified by PCR and were sequenced. Allele numbers for each loci were given according to the E. faecium MLST database (http://www.pubmlst.org/efaecium/). The combination of the allelic sequences for the seven genes yielded the allelic profile for each isolate. The goeBURST 1.2.1. algorithm was used for clustering the determined sequence types.

Epidemiological data analysis

Medical records of patients with VRE positive fecal samples were investigated 6 months prior screening for the following factors: age, sex, underlying disease, length of hospitalization, antibiotic consumption, intravenous catheter, dialysis, nasogastric tube, tracheostomy, ventilator use, absence of normal enteric flora and presence of toxin positive Clostridioides difficile. We analyzed our database (MedBakter) for any other VRE positive clinical specimen of the carriers 6 months preceding and following our screening. For statistical analysis we investigated 93 VRE negative fecal samples; patients in this population were selected by Monte-Carlo simulation based on random number generation.

Medical data was evaluated with the SPSS software version 24.0 (SPSS Inc.). The outcome of colonization with VRE was expressed as binary categorical variable. All categorical variables were analyzed by Chi-square test. If all expected value frequencies were not equal to or greater than 5, Fisher exact probability test was used while logistic regression was used for continuous independent variables and categorical outcome. P-value less than 0.05 was considered statistically significant. Additionally, odds ratio was used to determine the association between risk factors and VRE colonization.

Analysis of the association between Esp and biofilm formation

Chi-square (χ2) significance test was carried out to investigate the association between the presence of the esp gene and biofilm positivity; p-value≤0.05 was considered statistically significant. Contingency table analysis was used to infer whether the presence of the esp gene is correlated with biofilm formation. Additionally we used odds ratio test to determine the chance of biofilm production if the esp gene is present. Evaluation of data was performed with the SPSS software version 24.0 (SPSS Inc.).
RESULTS

Characterization of VRE strains isolated from clinical samples

During the 4-year research period a total of 7799 Enterococcus spp. were recovered from various clinical samples. Among all enterococci 73 grew on the VRE screening plates originating from 43 patients. Identification with the MALDI-TOF MS revealed that all VRE were Enterococcus faecium (VREfm) with high score values (>2.0). The majority of VREfm were recovered from wound/decubitus (26%) followed by urine (23.3%), blood and cannula (14-14%). The mean patient age was 57 years with almost equal gender distribution (males: 51.2%, females: 48.8%).

Results of antibiotic susceptibility testing

All isolates showed resistance to vancomycin, ampicillin and ciprofloxacin, followed by gentamicin (81.4%). 7% of the strains showed resistance to teicoplanin. All of them were susceptible to newer antibiotics such as linezolid, tedizolid, tigecyclin and daptomycin.

Detection of vancomycin resistance genes and virulence factors

Among the 43 VREfm isolates 40 harboured the vanB (93%) and 3 the vanA (7%) gene. The gene coding for the Esp protein was present in 7 (16.3%) isolates which were obtained from various invasive and non-invasive body sites such as blood, cannula, cerebrospinal fluid, bronchial washing, sputum, urine and decubitus. None of the isolates yielded a PCR product with the vanC, vanD, vanE, vanG, ace or agg specific primers.

Results of biofilm formation

40% of the isolates were biofilm producers. Of the 43 isolates none proved to be strong biofilm producer. Out of the 17 isolates that formed biofilm only 4 (24%) produced biofilm moderately while 13 (76%) were weak biofilm producers. According to the origin of the samples among the invasive 71.4% (5/7) while from the non-invasive 33.3% (12/36) produced biofilm. Based on the Chi-square test there was no statistically significant correlation between biofilm production and isolation site. Correlation was found between the presence of the esp gene and biofilm production (p=0.059) with 90% confidence. The contingency table analysis showed a weak relationship (C=0.28). According to the odds ratio test the chance of biofilm positivity is five times higher if esp is present. Two isolates encoded esp but did not form biofilm, while twelve isolates were biofilm producers with no virulence genes detected.

Results of epidemiological analysis

At the beginning of the study (2012 and 2013) all VRE were tested, from 2014 PFGE was restricted to all of the vanA positive isolates as well as to invasive samples. PFGE analysis revealed 9 PFGE types based on a similarity cut off value of ≥ 85%. The predominant pulsotype was ENTCO-062 and included 11 (vanB) isolates followed by ENTCO-075 (3 vanB, 1 vanA isolates) and ENTCO-151 (2 vanA isolates). 6 additional PFGE types were represented by a single isolate each.

MLST analysis was performed from 14 VREfm isolates and revealed 6 different sequence types. The predominant ST in this study was ST17 (6 vanB isolates). Less frequent types were ST117 (2 vanA, 1 vanB), ST203 (2 vanA), ST78 (1 vanB), ST412 (1 vanB) and ST364 (1 vanB). All could be assigned to the clonal complex 17 (CC17), a specific lineage associated with nosocomial E. faecium strains.
**Characterization of VRE strains isolated from stool samples**

During the first research period a total of 2050 samples were screened by solid screen. 313 samples yielded black colonies on the screen agar. However, only 266 isolates were selected based on their colony morphology on blood agar for MALDI-TOF measurements. In the second research period 971 samples were screened with enrichment broth. Among them 469 caused blackening of the broth and according to their macroscopic morphology 264 isolates were selected for MALDI-TOF analysis.

Altogether a total of 102 non-duplicate VRE were obtained from 93 patients. Identification with the mass spectrometer revealed that 71 VRE were *E. faecium*, the second most common species was *E. gallinarum* (n=23) followed by 5 *E. faecalis*, 2 *E. casseliflavus* and 1 *E. hirae*. Among the *E. faecium* isolates 33 were vanA and 38 were vanB positive. Only 4 *vanA* and 1 *vanB* positive *E. faecalis* were detected. The single *E. hirae* was found to be *vanA* positive. All *E. gallinarum* carried the *VanC1* and *E. casseliflavus* the *VanC2* genes.

*VanB* positive isolates showed high level resistance against vancomycin and sensitivity to teicoplanin whereas the *vanA* positive isolates displayed high level resistance against both antibiotics. All of the *vanC* strains had low level vancomycin resistance and were susceptible to teicoplanin. Fortunately, all isolates were susceptible to newer antibiotics such as linezolid, tigecycline and daptomycin. Quinupristin/dalfopristin resistance was found only in 4 *E. faecalis* isolates.

**Epidemiological features of VRE carriers**

VRE carrier’s mean age was 47 years. The mean length of hospital stay prior screening was 25 days. The most prevalent underlying disease was malignancy (22.6%) followed by diabetes and viral or bacterial infection (21.5%). Among the investigated risk factors absence of normal enteric flora was found in 24.7%. 18 carriers (19.4%) had toxin positive *C. difficile* detected at least once at the time of screening or in the previous 6 months.

Statistical analysis of 93 colonized and non-colonized patients showed that diabetes, normal flora absence, *C. difficile* positivity, longer hospital stay and advanced age were significantly associated with VRE colonization. The highest chance occurred in case of diabetes. The chance of VRE positivity was 12.46 times higher in these patients. 78.5% of the VRE colonized patients received at least one kind of antibiotic during the 6 months prior stool sampling. Among them metronidazole was the most commonly administered (47.3%) followed by cephalosporins (40.9%).

**Characterization of screening methods**

In the first period of investigation the solid screening medium detected 46 VRE (2.2%) from 2050 stool samples while in the second period broth enrichment identified 56 VRE (5.8%) from 971 stools. Interestingly, 1 vancomycin resistant *E. hirae* was also identified in the second period.
DISCUSSION

Previously our research group assessed the prevalence of VRE between 2004 and 2009 at the teaching hospitals of the University of Debrecen and found the predominance of VanC positive \textit{E. gallinarum} and \textit{E. casseliflavus} strains. Since then the picture has changed. Recent data from the ECDC revealed a significant increase of invasive \textit{VREfm} between 2012 and 2018. In accordance with the national trends we observed the elevation of invasive and non-invasive \textit{VREfm} from 1.7\% to 11\% at our teaching hospital.

Fortunately, the newest antibiotics were the most effective agents against our isolates. The gene coding Ace did not occur in any of the tested strains. This is in agreement with previous findings of other research groups who found \textit{ace} only in \textit{E. faecalis}. Similarly, none of the tested \textit{VREfm} isolates harbored the gene for Agg in correlation with earlier reports. In our study, \textit{esp} was the only virulence factor found in 16.3\% of the strains. Several previous studies have presented that \textit{esp} is a signal for widely disseminated hospital clones of antibiotic-resistant \textit{E. faecium}.

Our research group evaluated biofilm formation and its relationship with virulence genes in \textit{E. faecium}. Association between the presence of the \textit{esp} gene and biofilm positivity was weak. When \textit{esp} is present in the strain the chance of biofilm positivity is five times higher. However, biofilm formation may rely on several different mechanisms independent of \textit{esp}.

PFGE analysis showed 9 different pulsotypes. 74\% of the strains could be grouped into 3 main clusters (ENTCO-062, ENTCO-075, ENTCO-151) indicating monoclonality. At the beginning of our survey ENTCO-062 pulsotype predominated but it was still present in 2014 and 2015 to a lesser extent. Although 5 isolates showed 100\% similarity within this cluster (with ST17 and \textit{vanB} genotype) these proved to be sporadic cases. At the end of the examination period rising prevalence of ENTCO-075 pulsotype was documented.

By MLST 14 \textit{VREfm} isolates could be assigned to six STs all belonging to clonal complex 17 and four of them (ST17, ST117, ST78, ST203) representing major ampicillin-resistant hospital-derived clones. Our research group was the first who described the \textit{VREfm} isolates belonging to ST412 and ST364 sequence types in the Central European region. An interesting finding was the detection of ST364. To our knowledge this ST was first reported by Zhu X et al. from Beijing, China and only one isolate was reported from Taiwan. Until now no other strain of this type has been deposited to the \textit{E. faecium} MLST database (http://www.pubmlst.org/efaecium/) from Eastern-Europe. ST412 has yet been found in several countries, mainly from the Americas and Western Europe. Interestingly, two isolates arose from blood with the same ST (ST117) and both were negative for \textit{esp} but one was \textit{VanA} and the other \textit{VanB} positive. Further analysis revealed that isolation time of the two strains was very close (within 5-days) but they were from different Departments. In these cases acquisition of either \textit{vanA} or \textit{vanB} gene clusters might have occurred in two different occasions.

Up till now there has been no information on fecal colonization rates with VRE of patients admitted to our hospital, despite the fact that carriers can serve as source of nosocomial infections. In my research we aimed to assess gastrointestinal VRE carriage rates in two periods in 2016 and found rates of 2.2\% with the same screening medium used in routine culturing and 5.8\% with enrichment broth.

Medical records of 93 VRE carriers were analyzed; 24.7\% of their routine culturing revealed absence of coliform bacterial normal flora. 18 were \textit{C. difficile} positive but only 12 (66.7\%) received vancomycin therapy. 17 VRE carriers received vancomycin but did not have a history of \textit{C. difficile} positivity. The association between \textit{C. difficile} positivity and VRE colonization was statistically significant (p=0.0345) likewise in other studies. The chance of VRE positivity is 2.55 times higher in CDI patients. In vancomycin receiving patients the chance of VRE positivity is 13.59 times higher than in case of no vancomycin therapy. In
conclusion, there is a 5.39 higher chance of becoming a VRE carrier after vancomycin therapy than after *C. difficile* infection.

Compared to previous reports we also found malignancy as the most prevalent underlying disease (22.6%) in VRE carrier. In 21.5% of our VRE carriers there wasn’t any other underlying disease documented in their medical history than a diagnosis related to infectious origin. It is obvious that infections have great impact on hospital stay and the chosen antibiotic therapy. Longer hospitalization results in extended antibiotic consumption and only 21.5% of the carriers did not receive any antibiotics. The anti-anaerobic drug metronidazole was the most commonly administered (47.3%) followed by cephalosporins (40.9%). 31.2% of carriers received vancomycin which was the fourth most commonly administered drug together with carbapenems.

In one interesting case the patient colonized with *vanA E. hirae* had previously *vanA E. faecium* isolated from his stool one month before. We assume that transfer of the *vanA* gene might have occurred between the two species, although we did not confirm this hypothesis with molecular methods. Overall, we detected 37.3% *vanA* VRE which is alarming since *vanA* can be transmitted to other nosocomial pathogens from a different genus such as *Staphylococcus aureus*.

Our intestinal VRE isolates were found to be sensitive to newer agents. Only 4 out of 5 *E. faecalis* isolates were resistant to quinupristin-dalfopristin (Q/D). Although, *Enterococcus faecalis* is intrinsically resistant to Q/D because of the expression of the *lsa* gene, clinical isolates with nonsense mutations in this gene can be susceptible.

Prevalence of colonization with VRE reported by several studies is difficult to compare due to the differences in the target population, sample types, detection methods using different media and/or glycopeptide concentrations. D’Agata and colleagues published that rectal swab failed to detect a large proportion of patients with gastrointestinal colonization. Therefore we decided to use stool samples in our study. Several articles have documented that broth enrichment enhances the detection rates. The limitation of our study was the investigation of two periods with two different methods, so direct comparison of solid medium and enrichment broth could not be carried out. Although more VRE have been identified with broth enrichment, the use of solid Screen is less labor-intensive and time-consuming, and therefore easier to implement in routine diagnostics.
SUMMARY

The aim of our surveillance study was to characterize and elicit the genetic relatedness of VRE isolated from clinical samples and to determine the carriage rate of VRE in stool samples. We also investigated various risk factors which could be associated with VRE colonization.

Between 2012 and 2015 we determined the presence of 40 VanB and 3 VanA E. faecium isolates. Fortunately, all of them were susceptible to newer antibiotics such as linezolid, tedizolid, tigecyclin and daptomycin. According to MLST all of the tested isolates belonged to clonal complex 17 (CC17), a specific lineage associated with nosocomial E. faecium strains. We also reported VREfm isolates belonging to ST412 and ST364 sequence types for the first time from the Central European region. PFGE revealed 9 different pulsotypes. 74% of the strains could be grouped into 3 main clusters (ENTCO-062, ENTCO-075, ENTCO-151) indicating monoclonality. Although 5 isolates showed 100% similarity within the ENTCO-062 cluster (with ST17 and vanB genotype) these proved to be sporadic cases.

We showed the presence of fecal carriage of VRE in the Eastern region of Hungary. Stool colonization proved to be 2.2% with solid screening medium but 5.8% with enrichment broth. Finding similar species and similar rates of vanA and vanB strains in stool VRE compared to clinical VRE might indicate the stool origin of the latter. Interestingly we found a high rate of fecal carriage of E. gallinarum which was present in clinical samples only till 2009 and was subsequently replaced by more resistant vanA/vanB strains.

In view of our findings we suggest limiting the inadequate use of antibiotics inefficient against enterococci and advise the screening of risk groups such as patients with malignancy, diabetes, history of recent hospitalization or former C. difficile infection. Fortunately, there was no outbreak during the investigation period but constantly high prevalence of MRSA and the increasing rate of C. difficile infections in Hungary can potentially lead to the increased use of vancomycin. Therefore we can expect the further rise of VRE especially in the nosocomial setting. Emergence of VREfm emphasizes the need for the application of stringent control measures to decrease the risk of dissemination of the resistant bacteria such as isolation of infected/colonized patients, increased environmental cleaning and improved antimicrobial interventions.
List of publications related to the dissertation

   IF: 1.448 (2016)

   DOI: http://dx.doi.org/10.1089/mdr.2018.0074
   IF: 2.397
List of other publications


Total IF of journals (all publications): 3,845
Total IF of journals (publications related to the dissertation): 3,845

The Candidate’s publication data submitted to the IDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

28 October, 2019
List of major presentation
