DETECTION OF ANTIBIOTIC RESISTANCE IN ENTEROCOCCI FROM HUMAN GUTS

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Abstract - We investigated the presence of antibiotic resistance genes in enterococci isolates that were obtained from faecal samples of 3 hospitalized patients receiving antibiotic prophylaxis therapy, and from ileostomy effluent of 1 subject without antibiotic dosage. 16S rRNA gene sequencing and (GTG)-5 PCR were used for classification of *Enterococcus* spp. Minimal Inhibitory Concentration (MIC) of vancomycin and double diffusion test for erythromycin resistance were determined. The carriage of vancomycin (*van*A, *van*B, *van*C1, *van*C2/C3, *van*D, *van*E and *van*G) and erythromycin (*erm*A, *erm*B, *erm*C and *mef*A/E) resistance genes were investigated by PCR. Of 63 isolates (19 from faecal samples and 44 from ileostomy effluent), 13 (21%) were identified as *E. faecium*, 29 (46%) *E. faecalis*, 5 (8%) *E. gallinarum* and 16 (25%) *E. avium*. Only *E. gallinarum* and *E. avium* were found in ileostomy samples. A total of 59 isolates (94%) were resistant to vancomycin; however, only *van*C1 gene was found in isolates (*E. gallinarum*). The phenotype found in all faecal isolates and in 13 (30%) of the ileostomy isolates corresponded to constitutive phenotype (cMLSb) erythromycin. *Erm*B genes were identified in 6 isolates from feces and 12 from ileostomy (*E. avium* and *E. gallinarum*). Additionally, these enterococci populations can be reservoirs for antibiotic resistance.

Key words - antibiotic resistance; vancomycin; erythromycin; *Enterococcus* spp.; humans.

1. Introduction

The genus *enterococcus* represents indigenous members of the human gut microbiota, they inhabit the gastrointestinal tract, the oral cavity and the vagina in humans as normal commensals, adapt to the nutrient-enriched, oxygen-depleted and ecologically complex gut environment [1]. The main members of enterococcal infections in humans are *Enterococcus faecalis* and *Enterococcus faecium*, which are the most common species in the intestinal human and animals e.g. cattle, pigs, fowl. Other enterococci species can cause infectious diseases in humans and animals including *Enterococcus avium*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus raffinosus*, and *Enterococcus mundtii* which occur not too frequently [2]. However, because of the increasing of antibiotic resistance and evasion or inhibition of immune responses of host, enterococci become recognized as nosocomial pathogens that can lead to a variety of seriously infectious diseases such as endocarditis, bacteraemia, meningitis, wound and urinary tract infections [3]. In recent decades, nosocomial infections in humans caused by enterococci have emerged and their incidence has rapidly been increasing in many countries [4]. In addition, most infections are opportunistic, and the diseases become difficult to treat because of the high rates of antibiotic resistance [5].

Enterococci are intrinsically resistance to beta-lactam antibiotics because they contain penicillin-binding proteins (PBPs). Besides, enterococci are also intrinsically resistant to low level of penicillinase-susceptible penicillin, penicillinase-resistant penicillins, cephalosporins, nalidixic acid, aztreonam, macrolides, and low levels of clindamycin and aminoglycosides [6]. Enterococci can acquire antimicrobial resistance through the transfer of resistance-encoding genes carried on mobile elements e.g. plasmids or transposons, or through the mutation to penicillin by beta-lactamases, chloramphenicol, tetracyclines, fluoroquinolones, high level of aminoglycosides, macrolides, and glycopeptides [7]. Glycopeptide and macrolide antibiotics have been used in the treatment of the infections caused by enterococci as well as other Gram-positive bacteria. Moreover, these antibiotics can be used for the replacement in patients who have resistance or allergy to penicillin or beta-lactam. However, the worldwide development of the resistance to glycopeptides and macrolides because of the use or misuse of antimicrobial drugs leading to intrinsic or acquired resistance to or tolerance of many antibiotics by mutation or by acquisition of plasmids or transposons, or bacterial chromosome containing genetic sequences that confer the resistances, has sometimes constrained the use of these antibiotics to certain indications, and these resistances may be make the antibiotic therapies become ineffectiveness [8].

There are many types of vancomycin-resistance Enterococci (VRE) characterized on phenotypes and genotypes. Generally, there are six most common VRE phenotypes as vanA, vanB, vanC (i.e. vanC1, vanC2/3), vanD, vanE, and vanG, besides there are others such as vanX, vanY, vanH [9]. The vanA phenotype displays resistance to high level of vancomycin (MIC $\geq$ 64μg/ml) and teicoplanin (MIC $\geq$ 8μg/ml); the vanB phenotypes are moderate resistance to vancomycin, from 4 to 1024 μg/ml, and they are susceptible to teicoplanin. The vanA and vanB phenotypes are most frequent in *E. faecalis* and *E. faecium*. The vanC phenotype was described firstly in *E. gallinarum*, and then in *E. casseliflavus* species. This phenotype possess the intrinsic low-level of resistance to vancomycin and susceptible to teicoplanin. The vanD phenotype found in *E. faecium* is characterized by moderate resistance levels to vancomycin and low-level resistance to teicoplanin. The vanE and vanG phenotypes correspond to low- and moderate-level resistance to vancomycin, respectively, but both of them are sensitive to teicoplanin in *E. faecalis*. The *van*A and *van*B operons are located on plasmids or chromosome; whereas the *van*C, *van*D, *van*E, and *van*G have been found exclusively on the chromosome [10]. Resistance to macrolides can spread from animal to human by spreading of the resistant bacteria or by horizontal gene transfer of mobile DNA elements containing the resistance genes. The resistance is caused by methylation of the ribosomal target of the antibiotics,

which leads to cross-resistance to macrolides, lincosamides, and streptogramins B, the so-called MLSb phenotype. The most common mechanism of the MLSb resistance is by rRNA methylases that methylate the adenine residues, encoded by the *erm* (erythromycin ribosome methylation) gene. The methylated adenine prevents the binding of the antibiotics to the 50S rRNA subunit. The expression of *erm* genes can be constitutive or inducible, and the presence of *erm* genes leads to high-level resistance to MLSb phenotype (MIC ≥ 128µg/ml). Several classes of *erm* genes have been described such as *erm*A, *erm*B, *erm*C, *erm*D, and so on, but in enterococci the *erm*B gene is the most frequently spread [11].

The present study aimed to determine antibiotic resistance genes in *Enterococcus* spp. isolates from faecal samples of hospitalized patients received antibiotic prophylaxis treatment, and from ileostomy effluent of human without antibiotic therapy in Netherlands.

2. Materials and methods

2.1. Bacteria collection and culture conditions

Enterococci isolates were obtained from two sources: 44 from ileostomy effluent of healthy human and 19 from faecal samples of intensive care units (ICU) patients in Netherlands with two different antibiotic prophylaxis therapies i.e. SDD-Selective Digestive Decontamination getting Tobramycin (80mg), Polymyxin (100mg), Cefotaxime (4x 1000mg), Amphotericin B (500mg) and SOD - Selective Oropharyneal Decontamination receiving Tobramycin (2%), Polymyxin (2%), Amphotericin B (2%). To differentiate enterococci from nonenterococcal group D Streptococci, colonies were transferred from Brain Heart Infusion (BHI) agar to Bile-esculin agar (BEA).

2.2. Identification and classification of isolates

All isolates were identified and classified by (GTG)5-PCR fingerprinting which can differentiates microorganisms by using primers complementary to interspersed repetitive consensus sequences. An electrophoresis gel was used for the analysis using BioNumerics software in Laboratory of Microbiology-Wageningen University & Research Center, Netherlands. Simultaneously, the sequences of the chromosomal DNA of isolates identified by 16S rRNA sequencing were used for the classification of BioNumerics analysis which is based on Pearson's similarity coefficient. The dendrogram construction was performed based on study of Gevers *et. al*. [5].

2.3. Antimicrobial susceptibility

The resistance of 63 enterococci isolates from ileostomy effluent and faeces to vancomycin was performed by dilution agar test on BHI agar containing 10µg/ml vancomycin. Positive growths indicated that the bacteria are resistant to vancomycin based on he minimal inhibitory concentration standard of CLSI Publishes 2012 Antimicrobial Susceptibility Testing Standards.

The resistance to erythromycin was performed by double disk diffusion test, which is to determine the phenotype of inducible clindamycin resistance. The isolates were grown on Mueller-Hinton (MH) agar in the presence of erythromycin (15µg/ml) and clindamycin disk separated by 20mm of distance. The presence or absence of growth around the antibiotic disks is an indirect measure of the ability of that drug to inhibit the organism. As erythromycin would act as an inducing agent, isolates carrying the *erm* gene will grow in resulting in a D-shaped zone around the clindamycin disk. These isolates should be reported as resistant to clindamycin [12].

2.4. DNA extraction

DNA isolation was performed by the protocol for gram-positive bacteria of QIAamp® DNA Mini Kit (Qiagen) [4].

2.5. Detection of antibiotic resistance genes

Multiplex PCR was used for the detection of the vancomycin-resistance genes *van*A, *van*B, and *van*C; single PCR for *van*D, *van*E, and *van*G, and the erythromycin-resistance genes including *erm*A, *erm*B, *erm*C. The *mef*(A/E) gene was detected by restriction analysis using *BamH*I, which discriminates between the *mef*(E) gene no recognition size of *BamH*I and the *mef*(A) gene containing the restriction site of *BamH*I. It will cut the fragment of *mef*(A/E) to 2 fragments 284bp and 64bp [2, 5].

3. Results and discussion

3.1. Results

3.1.1. Identification of isolates from faeces

A total of 19 isolates from faecal sample were identified and classified to the species level. The combination between results from rep-PCR with (GTG)5 primer (Figure 1) analyzed by BioNumerics software and the identification from 16S rRNA sequencing was made in order to get the reliable results of identification and classification of isolates including 9 (47%) isolates are *E. faecalis* and 10 (53%) isolates are *E. faecium*.

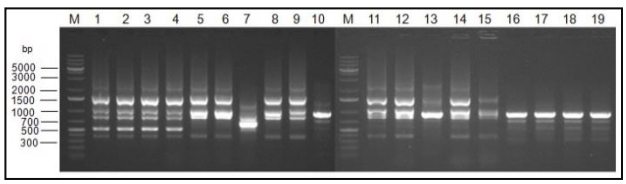

Figure 1. *Electrophoresis of (GTG)5-PCR product. M: DNA ladder 1kb plus, lanes with number from 1 to 19: Products of (GTG)5-PCR of DNA of 19 isolates from faecal samples. In this experiment, negative control is the master mix of PCR without DNA; positive controls are isolates signed 1, 2, 3, and 24 from ileostomy, which are already identified, all control are not shown on this picture*

3.1.2. Antibiotics resistance

Overall, high prevalence of resistance to vancomycin and erythromycin was detected (Table 1). Of 44 isolates from ileostomy effluent, 40 isolates were resistant to vancomycin (10µg/ml) corresponding to 2 *E. faecium*, 21 *E. faecalis*, 5 *E. gallinarum*, and 12 *E. avium*. From 19 faecal isolates, all were resistant to vancomycin (10µg/ml), corresponding to 9 *E. faecalis* and 10 *E. faecium*.

For the double disk diffusion test (phenotypes are described on Figure 2), 13 isolates from ileostomy effluent including 2 *E. faecium*, 5 *E. gallinarum*, 6 *E. avium* and all 19 isolates including 10 *E. faecium* and 9 *E. faecalis* from

faecal samples were resistance to both erythromycin and clindamycin. This result indicated the presence of constitutive (cMLSb) phenotype.

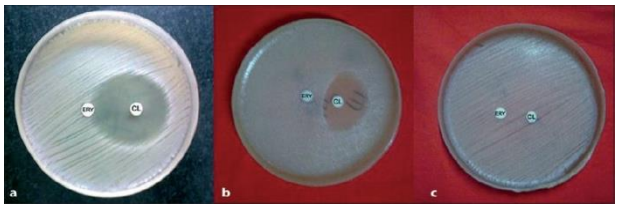

Figure 2. *Phenotype of erythromycin-resistance. (a) M phenotype: isolates show the resistance to erythromycin (zone size ≤ 13mm) while sensitive to clindamycin (zone size ≥ 21mm) and give circular zone of inhibition around clindamycin disk, (b) iMLSb phenotype: isolates show the resistance to erythromycin (zone size ≤ 13mm) while being sensitive to clindamycin (zone size ≥ 21 mm) and give a D-shaped zone of inhibition around clindamycin with flattening towards erythromycin disk, (c) cMLSb phenotype: isolates show resistance to both erythromycin (zone size ≤ 13mm) and clindamycin (zone size ≤ 14mm) with circular shape of zone of inhibition if any around clindamycin disk [2]*

Table 1. *Antibiotic resistance profile of enterococci isolates*

Antibiotic	Number of strains enterococci				
	Faecium	*Faecalis*	*Gallinarum*	*Avium*	Total
Vancomycin	12	30	5	12	59
Erythromycin	12	9	5	6	32

3.1.3. Molecular detection of antibiotic resistance gene

Multiplex PCR for the vancomycin-resistance genes indicated the presence of *van*C1 gene in 5 *E. gallinarum* (12.5%) from ileostomy effluent. No vancomycin-resistant genes detected from isolates of faecal samples. Single PCR were performed to detect the erythromycin resistance genes (*erm*A, *erm*B and *erm*C), only the *erm*B gene were detected. PCR results revealed that 12 of 13 erythromycin-resistant isolates (92.31%) from ileostomy effluent carry the *erm*B gene in which 2 *E. faecium*, 5 *E. gallinarum*, and 5 *E. avium*. At the meanwhile, 6 of 19 erythromycin-resistance isolates from faecal samples carried the *erm*B gene corresponding to 4 *E. faecalis* and 2 *E. faecium* (Figure 3).

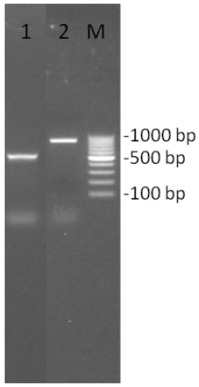

Figure 3. *Electrophoresis for the detection of ermB gene 639bp (lane 1) and vanC1 gene 822bp (lane 2). M: DNA ladder 100bp*

After that, 14 cMLSb phenotype isolates i.e. 1 *E. gallinarum* from ileostomy effluent, 9 *E. faecium* and 4 *E. faecalis* from faecal samples, that were negative to the occurrence of *erm*B gene, were used to detect the presence of *mef*(A/E) gene by restriction analysis. However, no *mef*(A/E) gene was determined in these isolates (Table 2).

Table 2. *The number of resistance genes among isolates*

Deteced genes	Number of strains enterococci				
	Faecium	*Faecalis*	*Gallinarum*	*Avium*	Total
*van*C1			5		5
*erm*B	4	4	5	5	18

3.2. Discussion

In the present study, 19 isolates from faecal samples of ICU patients with antibiotic prophylaxis therapies were identified and classified by 16S rRNA sequencing and (GTG)5-PCR; in addition, we included 44 isolates from ileostomy effluent previously identified and classified as *enterococcus* species; in order to analyse in both group the antibiotic resistance and the presence of virulence factors.

By using the (GTG)5-PCR fingerprinting technique, 19 isolates from faecal samples of ICU patients were identified and classified. All these isolates were grouped in separated clusters according to their respective taxonomic designations on 16S rRNA sequencing, and the obtained results from (GTG)5-PCR fingerprinting classified completely the previous prediction. The BioNumerics software analysis was used to analyse the pattern and differentiate groups of enterococci species based on the repetitive of the same pattern with the high qualitative intensity in different samples. The complexity of the (GTG)5-PCR bands was not the same for all isolates; the number of patterns of different major groups were not repeatedly the same including in 2 groups of *E. faecium* and *E. faecalis*, they had quite equal number of bands. This observation may be a disadvantage for the identification and classification of enterococci strains, but this method has a high sensitivity to small genomic variations. Therefore, the loss or addition of one genomic (GTG)5 element can change the DNA pattern; as a result, it differentiated closely related species in separated clusters, and classified enterococci species which have been discriminated inconclusive by 16S rRNA sequencing. Similar results had been obtained by Svec *et. al.* [7] who used (GTG)5-PCR for identification and classification of *Enterococcus* spp. In addition this technique has been already applied successfully in identification and classification of not only enterococci species, but also several bacteria species, such as *Lactobacillius* species by Gevers *et. al.* [5], acetic acid bacteria by De Vuyst *et. al.* [6], or *Staphylococcus* spp. by Svec *et. al.* [7].

The resistance to glycopeptide was tested by dilution agar test using vancomycin (10µg/ml), one of the principal antibiotics in this group. So, from ileostomy effluent, the resistance was occurred on 4 species of enterococci i.e. *E. faecium, E. faecalis, E. gallinarum*, and *E. avium*; and from faecal samples, all isolates (*E. faecium* and *E. faecalis*) were resistant to this antibiotic. Similar results were found by Iris *et. al.* [15] or by indicating a high significant rate of vancomycin-resistant enterococci colonization isolated from rectal swabs of patients in ICU, or by Mioljevic *et. al.* in ICU patients from a clinical center of Serbia, Belgrade [14], and by Babar *et. al.* in a tertiary care hospital [8]. Multiplex PCR (for *van*A, *van*B, *van*C1, *van*C2/3) and

single PCR (for *van*D, *van*E, *van*G) were performed to detect responsible genes for vancomycin-resistant phenotype. The *van*C1 gene was detected in *E. gallinarum* isolates from ileostomy effluent. Different enterococci species can carry different vancomycin-resistance genes, as is indicated by previous study of Satake *et. al.* [9]. From faecal samples, the *van*A gene has been detected in a wide variety of enterococcal species; the *van*B gene often occurs on *E. faecium* and *E. faecalis*; *van*C1, *van*C2, and *van*C3 genes have been recognized in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*, respectivel [9]. Patel and partners identified *van*C1 gene only in *E. gallinarum* from one hundred of clinical isolates of *Enterococcus* spp. [13]. Fifteen years later, Dombradi and co-workers determined the presence of *van*C1 gene exclusively in *E. gallinarum* isolated from clinical samples by multiplex PCR [10]. Hence the emergence of *van*C1 gene is likely to be more common in *E. gallinarum* that is relevant to our result. Although most of evidence indicated that *E. gallinarum* carried the *van*C1 gene encoding for the resistance to low level of vancomycin, sometimes this enterococcus species also carries other genes encoding high level resistance to vancomycin. Dutka *et. al*. indicated that *E. gallinarum* isolates can be resistant to high level of vancomycin (MIC > 256µg/ml) due to the presence of both *van*A and *van*C1 genes [11]. In our study, *van*A and *van*B genes, which are more frequently vancomycin-resistance genes, were not detected in vancomycin-resistant isolates, it is possible that, in addition to the problem of detection, the localization of the genes cluster responsible for *van*A and *van*B resistance is in transposable elements, and in some cases the high level resistant is associate to glycopeptide dependence mean mutant *enterococcus* that actually require these agents to their growth [14].

The resistance to erythromycin antibiotics was determined by double disk diffusion test; accordingly, only the constitutive erythromycin resistance phenotype (cMLSb) was detected in 13 isolates from ileostomy effluent (*E. faecium*, *E. gallinarum* and *E. avium*) and 19 isolates from faecal samples (*E. faecium* and *E. faecalis*). Similar results were reported by Bouchami *et. al.* in *E. faecium* isolates from neutropenic patients hospitalized at different units of the Bone Marrow Transplant centre of Tunisia, in addition they found the inducible phenotype (iMLSb) in only one isolate. Then, PCR was performed to detect the genes responsible for this phenotype; the *erm*B gene was present in 56% of erythromycin-resistant isolates corresponding to *E. faecium* (11%), *E. gallinarum* (28%) and *E. avium* (28%) from ileostomy effluent and *E. faecium* (11%) and *E. faecalis* (22%) from faeces. Previous studies indicated that the high prevalence of erythromycin resistance reported corresponding to the MLSb phenotype which is encoded mainly by the *erm* genes, with the *erm*B gene as the most frequently found erythromycin-resistance gene among enterococci [2]. Franz-Josef Schmitz and co-workers also found that *erm*B gene (93%) and followed by *erm*A gene (4%) encoded for the erythromycin-resistance in clinical isolates of *E. faecium* which displayed the cMLSb resistant phenotype [12]. These results indicated that *erm*B is the most frequently resistance gene among erythromycin-resistant enterococci performing MLSb phenotype; however, the *erm*B gene was not detected in some other erythromycin-resistant enterococci. Therefore, from the cMLSb phenotype isolates which were negative to *erm*B gene, the presence of *mef*(A/E) gene encoding for efflux mechanism was investigated, however no *mef*(A/E) gene was in these isolates. So, probably there is another mechanism of erythromycin resistance in these isolates e.g. efflux mechanism encoded by *mrs* gene, or inactivation mechanism of macrolides encoded by *mph* gene.

3.3. Conclusion

From a total of 63 isolates, 19 isolates from faecal samples of ICU patients receiving antibiotic prophylaxis therapies were identified and classified into 10 *E. faecium* and 9 *E. faecalis*. We included 44 isolates from ileostomy effluent of healthy humans previously identified as 21 *E. faecalis*, 2 *E. faecium*, 5 *E. gallinarum*, and 16 *E. avium*. Among these all isolates, there were 59 enterococci resistant to vancomycin based on dilution agar test, and 32 isolates resistant to erythromycin by double diffusion test.Based on the phenotypes of vancomycin and erythromycin resistance, the presence of antibiotic-resistance genes were detected by PCR method. Our results indicated the presence of *van*C1 gene in 5 *E. gallinarum* from ileostomy effluent; *erm*B gene in 12 isolates from ileostomy effluent including *E. faecium*, *E. gallinarum*, *E. avium* and 6 isolates from faeces corresponding to *E. faecium* and *E. faecalis*.

Additionally, the prevalence of glycopeptides and macrolides resistance in enterococci can be associated with enterococcal invasion and the spread of infectious diseases in humans.

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