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Molecular Genetic Profiling of Clinical and Foodborne Strains of Enterococci with High Level Resistance to Gentamicin and Vancomycin

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Abstract

Enterococci often acquire antimicrobial resistance through horizontal gene transfer. Relatedness between enterococci with high level resistance to gentamicin and vancomycin isolated from humans, food and hospital environment in Tandil County (Argentina) was investigated. PCR amplification for species determination was carried out. Resistance to seven antimicrobials was studied; virulence genes (*esp*, *cylA*), vancomycin and gentamicin resistance genes were investigated. In the isolates with high level antimicrobial resistance (gentamicin, vancomycin), pulse-field gel electrophoresis was performed. Vancomycin-resistant *E. faecium* (*n*:13) were recovered from human, food and hospital environment samples. All the isolates expressed high-level vancomycin and teicoplanin (*vanA* genotype), as well high-level gentamicin and streptomycin resistance. Vancomycin-resistant *E. faecium* were distributed among seven clonal types; esp gene was detected in clinical strains. There was no clonal relationship with food *vanA E. faecium*, but these strains could pose a risk in intra/inter genus transfer of *vanA* determinant to human-adapted strains. High-level gentamicin resistant *E. faecalis* (*n*:7) were recovered from human and food samples. Glycopeptide resistance was not observed; *cylA* gene was detected in most of the clinical high-level gentamicin resistant *E. faecalis* isolates. PFGE patterns showed four clonal types in high-level gentamicin resistant *E. faecalis* strains; there was demonstrated clonal relatedness between isolates from different origin. In Argentina, this is the first study showing a clonal relationship between high-level gentamicin resistant *E. faecalis* isolated from food and humans. These results encourage the study of dissemination of clonal complexes with mobile resistance genes.

Keywords: Enterococci; Relatedness; Food; Humans; Environment

Introduction

Enterococci are intrinsically resistant to many antimicrobials groups. Furthermore, these bacteria are capable of acquiring drug resistance genes through horizontal gene transfer, such as high level aminoglycosides and vancomycin resistance genes [1,2].

Enterococci colonize raw and fermented meat, as well as dairy products, since they are part of the indigenous microbiota of mammal's gastrointestinal tract and are able to survive under adverse environmental conditions [3,4].

Enterococcus faecalis and *Enterococcus faecium* have been recognized as etiological agents of bacteremia, meningitis, endocarditis, urinary tract and neonatal infections. Both species are relevant pathogens for immunosuppressed patients or with prolonged hospitalization [5-8].

Enterococci remain for long time-periods on environmental surfaces, including medical equipment, bed rails and doorknobs. Also, they are tolerant to heat, chlorine and some alcohol-based preparations [9].

In enterococcal species, vancomycin resistance can be associated with different *van* genotypes and Van phenotypes. *VanA* resistance is mediated by transposon Tn*1546* or closely related elements. Expression of *vanA* gene leads to inducible high-level vancomycin (Minimum Inhibitory Concentration, MIC, ≥ 64 µg/mL) and teicoplanin (MIC ≥ 16 µg/mL) resistance, while *vanB* gene encodes for variable levels of inducible vancomycin resistance. *VanA* is the most frequent glycopeptide resistance type in clinical enterococci. Nowadays, there is an increasing concern about *vanA* plasmid-mediated transfer to methicillin-resistant *Staphylococcus aureus* [10-12].

Over the last decades, detection of *vanA* genotype in *E. faecium* from animals and from food of animal origin has been reported. Initially, vancomycin resistance was linked with the use of avoparcin (glycopeptide) as an animal growth promoter in the European Union, EU [13-15]. After avoparcin was banned, glycopeptide-resistance persisted, likely due to co-selection processes. However, at the same time of avoparcin ban in the EU, vancomycin-resistant *E. faecium* were not isolated from animal food products in the US [16,17]. In Argentina, Delpech et al. [18] recovered vancomycin-resistant *E. faecium* from artisanal salami, cow cheese and goat cheese.

The incidence of health-care associated infectious diseases produced by vancomycin-resistant *E. faecium* in US hospitals has increased significantly, becoming a nosocomial pathogen almost as prevalent as *E. faecalis* [19].

Frequently, hospitalized patients are treated with broad spectrum antimicrobials. Therefore, presence of *VanA* or *VanB* vancomycin-resistant enterococci in patients' gastrointestinal tract and the risk of invasive infections can be significantly increased. In humans, *E. faecium* is the main reservoir of *VanA* and *VanB* type resistance. In recent years, worldwide, colonization with vancomycin-resistant *E. faecium* was considered endemic in many hospitals. Antimicrobial use in patients seems to be a critical factor related to vancomycin-resistant enterococci infections [20-22].

In South America, a multicenter study found that, ca. 22% of enterococcal infections were caused by multi-resistant *E. faecium*, similarly to what was reported in the US [23]. In Argentina, Corso et al. [24], through a nation-wide antimicrobial resistance survey, proved that most of vancomycin resistant enterococci carried *vanA* gene (98%). In addition, these strains, expressed high-level resistance to gentamicin (77.2%) and streptomycin (95.8%).

Many putative virulence factors from *E. faecium* have been identified. One of the most relevant is a large surface protein (Esp) covalently linked to the bacterial cell wall. Leavis et al. [25] demonstrated that, in *E. faecium,* esp gene was encoded in a large pathogenicity island and its presence was associated with nosocomial outbreaks.

In enterococci, acquisition of high-level gentamicin resistance (MIC ≥ 2,000 µg/mL) is a significant therapeutic problem, particularly for patients with severe infections. High-level aminoglycoside resistance makes ineffective the synergistic effect between aminoglycosides and a cell-wall-active agent, e.g. beta-lactams or vancomycin. *aac(6')-Ie-aph (2")-Ia* gene, associated with high-level gentamicin resistance, is widely spread in *E. faecalis*. This gene has been detected in strains from human infections and among enterococci from food of animal origin [26,27]. Bifunctional enzyme AAC6´-Ie-APH2´´-Ia confers resistance to available aminoglycosides, except for streptomycin. *aac(6')-Ie-aph(2")-Ia* gene is generally flanked by inverted repeats of IS256, making up composite transposons, such as Tn*5281* in *E. faecalis*. Fast dissemination of the genetic determinant led to consider the impact of its horizontal transferability among enterococcal species from different origin. Hence, human enterococcal isolates are not the only ones to be regarded as a reservoir of gentamicin resistance genes [28]. Among high-level gentamicin resistant enterococci, chromosomal *aph(2´´)-Ib, aph(2´´)-Id* and plasmidic *aph(2´´)-Ic* encode for gentamicin modifying enzymes as well [29].

Recently, our group studied antimicrobial resistance profiles in enterococci from minced meat, cow and goat cheese. In *E. faecalis* recovered from these products, high-level gentamicin resistance was detected [18].

Cytolysin is a chromosomal or plasmid encoded toxin with lytic activity against eukaryotic cells, helping enterococci to escape from host's immune response [30]. In human infections and experimental models, such as enterococcal endocarditis, cytolysin production has shown to contribute with virulence [31]. Huycke et al. [32] reported that ca. 60% of *E. faecalis* isolated from infection sites produced cytolysin and it was associated with a five fold-increased risk of acutely terminal outcome in patients with bacteremia. Moreover, high-level gentamicin resistant (HLGR)-cytolysin producer *E. faecalis* influence clinical and microbiological resolution of severe infectious diseases [33].

In Argentina, genomic relationship between vancomycin-resistant *E. faecium* and *E. faecalis* isolates with high-level gentamicin resistance from human and food origin has not been studied so far.

The aim of this study was to investigate the relatedness between HLGR *E. faecalis* and vancomycin-resistant *E. faecium* strains isolated from humans, food and hospital environment in Tandil City, Argentina.

Methods

Samples

Enterococci were recovered from the following sources: human, hospital environment and food of animal origin. Isolates were collected during the period January-December 2013, in Tandil County, at the Southeast region of Buenos Aires Province, Argentina. All isolates were stored, by triplicate, in brain heart infusion (BHI) broth with 30% glycerol at -70°C.

Human source: Samples for microbiological diagnosis of invasive infections caused by enterococci were collected from ICU´s (Intensive Care Unit) patients at Hospital Ramon Santamarina de Tandil (HRS).

Samples: Blood, cerebrospinal, abdominal, pleural and synovial puncture fluids. They were inoculated into BacT/ALERT culture bottles (BacT/ALERT® 3D Microbial Detection System, BioMérieux, Argentina). One significant isolate per patient, with an identifying number, was included in the study.

Hospital environment: Samples from surveillance cultures, in order to investigate the presence of vancomycin-resistant *E. faecium* in ICU-HRS. Study of ICU´s environmental contamination was performed investigating the surfaces close to patients (mattresses, bed rails and doorknobs) and medical instruments in contact with them. This procedure was carried out each time a vancomycin-resistant *E. faecium* was isolated from a patient. Samples were spread on bile esculin agar azide (BEEA) with 8 µg/mL of vancomycin and incubated for 48 h at 35°C. Each vancomycin-resistant *E. faecium* isolate from different places was labelled with a numerical identification code.

In all patients, admitted and discharged from the UCI, rectal swabs were done and processed as environmental samples.

Food: Artisanal meat and dairy products were purchased from 20 food retailers of Tandil County (ET1-ET20). In total, 146 samples (35 goat cheese, 32 cow cheese, 37 salami, 42 minced meat) were analyzed. Samples were sent (refrigerated at 4°C) to the Microbiology Laboratory and immediately processed.

Sampling and isolation techniques used have been previously described by Delpech et al. [26]. Additionally, enrichment in BHI broth with vancomycin (8 µg/mL) was performed for all samples. After incubation for 24 h at 35°C, 10 µL of each enrichment culture were spread on BEEA with vancomycin (8 µg/mL).

Susceptibility tests

MICs of all enterococcal isolates were determined by the agar dilution method, according to Clinical and Laboratory Standards Institute's (CLSI) recommendations [34]. The following antimicrobials, used in human medicine, were tested: ampicillin, ciprofloxacin, linezolid, vancomycin, teicoplanin, gentamicin and streptomycin. Quality control strains: *E. faecalis* ATCC 29212 (susceptible to vancomycin and gentamicin), *E. faecalis* ATCC 51299 (resistant to

gentamicin and vancomycin) and *E. faecium* ATCC 51559 (multidrug-resistant).

Phenotypic characterization was performed by Gram staining, catalase production, hydrolysis of pyrrolidonyl beta-naphthylamide, and growth in BHI broth with 6.5% NaCl. Species-level characterization was carried out by hydrolysis tests (arginine, pyruvate and methyl-α-d-glucopyranoside), tolerance to tellurite 0.04%, fermentation of carbohydrates (mannitol, arabinose, sorbitol, sucrose, raffinose, and sorbose), motility in thioglycolate broth, and agar pigment production [27].

Phenotypic confirmation was done using the Automated VITEK® 2 System (BioMérieux, Argentina).

Genomic DNA was extracted from phenotypically identified *E. faecalis* and *E. faecium* isolates by a previously described boiling method [35]. Molecular identification (polymerase chain reaction, PCR) was performed.

For genus confirmation, the protocol described by Ke et al. [36] was used. A *tuf* gene fragment (803 bp), encoding for a specific elongation factor of the genus *Enterococcus* was amplified (Table 1). One nanogram of genomic DNA was transferred directly to a 19 µL PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH: 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM each Enterococcus-specific primer, 200 mM each deoxynucleoside triphosphate, 3.3 mg/mL of bovine serum albumin and 0.5 U of Taq polymerase. The PCR mixtures were subjected to thermal cycling: 3 min at 96°C; then, 40 cycles of 1 s at 95°C (denaturation) and 30 s at 55°C (annealing-extension step).

Gene (type)	Nucleotide sequence (5'-3')	Annealing Temperature (°C)
tuf (Genus marker)	TACTGACAAACCATTCATGATG	55°C
	AACTTCGTCACCAACGCGAAC	
$ddl_{E.faecalis}$ (Species identfication)	ATCAAGTACAGTTAGTCT	54°C
	ACGATTCAAAGCTAACTG	
$ddl_{E.faecium}$ (Species identification)	TAGAGACATTGAATATGCC	54°C
	TCGAATGTGCTACAATC	
cylA (Virulence factor)	ATGGATGGGACAGATGGAAA	54°C
	AGCTGCGCTTAGTTCTGGAG	
esp (Virulence factor)	GGAACGCCTTGGTATG	58°C
	CCGCTTTTGGTGATTC	
vanA (Glycopeptide resistance)	GTAGGCTGCGATATTCAAAGC	54°C
	CGATTCAATTGCGTAGTCCAA	
vanB (Glycopeptide resistance)	GGTATCAAGGAAACCTC	54°C
	CTTCCGCCATCATAGCT	
aac(6')- Ie- aph(2")-Ia (Aminoglycoside resistance)	CAGGAATTTATCGAAAATGGTAGAAAAG	54°C
	CACAATCGACTAAAGAGTACCAATC	
aph (2´´)-Ib (Aminoglycoside resistance)	TATGGATTCATGGTTAACTTGGACGCTGAG	54°C
	ATTAAGCTTCCTGCTAAAATATAAACATCTCTGCT	
aph (2´´)-Ic (Aminoglycoside resistance)	GAAGTGATGGA AATCCCTTCGTG	54°C
	GCTCTAACCCTTCAGAAATCCAGTC	
aph (2´´)-Id (Aminoglycoside resistance)	GGTG GTTTTTACAGGAATGCCATC	54°C
	CCCTCTTCATACCAATCCATATAACC	

Table 1: Primers for identification of enterococci and antimicrobial resistance genes by PCR.

For species identification, PCR with species-specific primers: $ddl_{E.faecalis}$ (941 bp) and $ddl_{E.faecium}$ (550 bp), for the chromosomally encoded D-alanine: D-alanine (D-Ala:D-Ala) ligases was carried out. A final volume of 100 µL containing 250 ng of DNA as template; 50 pmol of each oligodeoxynucleotide primer; 500 mM (each) dATP, dCTP, dGTP and dTTP; 67 mM Tris-HCl (pH 8.8); 7 mM $MgCl_2$; 17 mM ammonium sulfate; 10 mM b-mercaptoethanol; and 2 U of Taq DNA polymerase. The cycles used were 94°C for 2 min for the first

cycle; 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for the next 30 cycles; and 72°C for 10 min for the last cycle [37].

For detection of the cytolysin encoding gene *cylA*, PCR was performed in a 25 µL total volume containing 20 pmol of each primer (517 bp fragment), 200 mM of each dNTP, 1 U of Taq polymerase, 1.5 mm $MgCl_2$, 2.5 mL of 10X PCR buffer and 2 µL of template DNA. Reaction settings: 2 min at 94°C (initial denaturation) followed by 30 cycles of 30 s at 90°C (denaturation), 30 s at 54°C (annealing) and 1 min at 72°C (extension). A final extension cycle was performed at 72°C for 8 min [27].

Enterococcal strains were screened for the *esp* gene by PCR, according to the protocol of Coque et al. [38]. Primers were used for amplifying an 800 bp gene fragment, encoding for *esp*: 5′-GGAACGCCTTGGTATG-3′ and 5′-CCGCTTTTGGTGATTC-3′. The reaction mixture consisted of 25 µL PCR master mix, 5 µL primers (2µM), 15 µL sterile water and 5 µL of bacterial DNA. PCR was carried out with the following thermal cycling profile: initial activation step at 95 °C for 15 min; 30 cycles of denaturation at 90°C for 30 seconds, annealing at 58°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 10 min.

Resistance genes were detected by PCR using specific primers for the aminoglycoside-modifying enzymes *aac(6′)-Ie-aph(2″)-Ia* (369 bp), *aph (2´´)-Ib* (867 bp), *aph (2´´)-Ic* (444 bp) and *aph (2´´)-Id* (641 bp) genes. Detection of high-level gentamicin resistance genes was done according to Sparo et al. [27]. PCR was performed in a final volume of 25 µL, with 20 pmol of each primer, 1-2 colonies of each bacterial isolate, 200 µM of each dNTP, 1 U of Taq polymerase, 1.5 µM $MgCl_2$, 2.5 µL PCR 10X buffer and 2 µL of template DNA. Setting: a first cycle, 2 min at 94°C; followed by 30 cycles of 30 s at 90°C, 30 s at 54°C and 1 min at 72°C. A final extension cycle for 8 min at 72°C was done.

PCR for detection of *vanA* and *vanB* genes fragments (732 bp and 625 bp, respectively), which encode for D-Ala:D-Ala ligases of altered substrate specifity, was carried out according to the same protocol of Dutka-Malen et al. [37] for detection of *ddl* genes.

PFGE typing

Bacterial isolates were grown overnight in BHI broth. Chromosomal DNA was prepared in agarose plugs, digested with the restriction enzyme *Sma*I. DNA fragments were separated in a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA), following the protocol of Corso et al. [24]. *Sma*I was chosen upon the base composition (%G+C content) of the DNA of the microorganisms in study. In the case of low GC bacteria, as *E. faecalis* and *E. faecium*, *Sma*I (CCCGGG) is preferred, since it is advisable to use enzymes which recognize relatively few sites on the genome and give a resolvable and informative number of DNA fragments on the PFGE gel. Lambda ladder (New England Biolabs, Beverly, MA, USA) was used as molecular size standard. Similarity between isolates was determined by visual comparison of isolates DNA banding pattern. Interpretation of band patterns was carried out according to Tenover et al. [39].

Isolates were defined as distinct strain types, or unrelated, if their PFGE patterns differed by more than six bands. Types were named using a capital letter following the alphabet order (e.g. type A, B, C, D). Subtypes were defined as strains that differed by 2-6 bands, which were considered closely or possibly related, and were named using an Arabic number (e.g. subtype D1, D2) following the capital letter. Those isolates whose restriction patterns had the same number and size of bands were considered genetically indistinguishable and were assigned to the same strain type.

Results

Vancomycin-resistant *E. faecium* were isolated from samples from different sources: human, hospital environment and food of animal origin. Food enterococci were recovered from samples collected at five retailers (ET1, ET2, ET3, ET4, ET5).

Clinical vancomycin-resistant *E. faecium* were isolated from six (*n*=6) patients with invasive infections, five from hemoculture and one from abdominal puncture fluid (Figure 1). Also, vancomycin-resistant *E. faecium*, were recovered from *n*=3 rectal swabs from patients without enterococcal invasive diseases and *n*=1 in a mattress of a patient (HRSER460) near other one with a positive rectal culture.

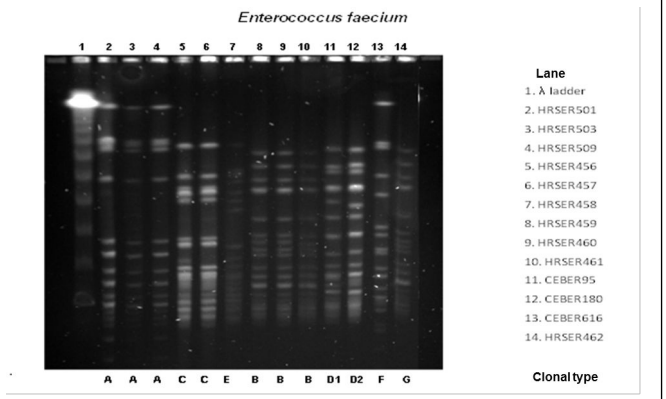

Figure 1: *Sma*I-PFGE patterns in vancomycin-resistant *Enterococcus faecium* isolates (Lane 1: lambda ladder; Lane 2: *E. faecium* EHRSER501 (human); Lane 3: *E. faecium* HRSER503 (human); Lane 4: HRSER509 (human); Lane 5: HRSER456 (human); Lane 6: HRSER457 (human); Lane 7: HRSER458 (human); Lane 8: HRSER459 (environment); Lane 9: HRSER460 (human); Lane 10: HRSER461 (human); Lane 11: CEBER95 (food); Lane 12: CEBER180 (food); Lane 13: CEBER616 (food); Lane 14: HRSER462 (human)).

Three vancomycin-resistant *E. faecium* were isolated from food: handmade "salame", cow cheese and sheep cheese from ET1, ET3 and ET5, respectively. In all vancomycin-resistant *E. faecium* (*n*=13), vanA gene was detected and MICs were as follow: vancomycin ≥ 256 µg/mL; teicoplanin ≥ 256 µg/mL; linezolid ≤ 2.0 µg/mL; streptomycin >2,000 µg/mL. Moreover, human and hospital environment vancomycin-resistant *E. faecium* were resistant to ampicillin (MIC ≥ 32 µg/mL), ciprofloxacin ≥ 8.0 µg/mL and showed high-level resistance to gentamicin (MIC > 500 µg/mL). In contrast, vancomycin-resistant *E. faecium* isolates from food were susceptible to ampicillin (MIC ≤ 2.0 µg/mL), ciprofloxacin (MIC 1.0-2.0 µg/mL) and without high-level gentamicin resistance (MIC < 500 µg/mL).

Vancomycin-resistant *E. faecium* isolates could be differentiated in seven clonal types: VRE- A, VRE- B, VRE-C, VRE-D, VRE-E, VRE- F and VRE-G. Clone A, were detected from two blood culture isolates, from patients HRSER501 and HRSER503 with invasive infections and other patient (HRSER509) with rectal colonization; all of them hospitalized in ICU for the same period. Clone B included three

isolates, a positive hemoculture from patient HRSER461 and an environment contamination of a mattress following the discharge from ICU of patient HRSER459 and a rectal colonization of a patient (HRSER460) next to him; both without enterococcal invasive disease. Clone C was represented by 2 clinical isolates from hemoculture and an abdominal puncture fluid (patients HRSER456 and HRSER457), admitted in ICU at different time. Clone D was identified in two isolates of food, "salame" and sheep cheese, from different industrial plants (ET1 and ET4), not produced in the same period. Those isolates were identified as belonging to two subtypes (D1, D2) and considered as closely related. Clonal types E (hemoculture), F (cow cheese) and G (rectal swab) were represented by a single isolate each.

Only hospital vancomycin-resistant *E. faecium* isolates carried *esp* gene, excluding clonal type VRE-G.

During 2013, from 10 patients were recovered *E. faecalis* producing invasive infections and 4/10 of them showed high-level gentamicin resistance. Regarding food of animal origin, *E. faecalis* was isolated from different samples: minced meat for hamburgers, 5; regional handmade "salame", 2; sheep cheese, 4; cow cheese, 3. HLGR *E. faecalis* (3/14) were recovered from minced meat for hamburgers elaborated in ET4 (2/3) and "salame" (1/3), manufactured in ET1. Those isolates were recovered in different seasons: summer (January) and fall (April).

All *E. faecalis* isolates expressing high-level gentamicin resistance carried the *aac(6')- Ie- aph(2")-Ia* gene. None showed multiple antimicrobial resistance. Determined MICs were: gentamicin > 500 µg/mL; streptomycin ≤ 500 µg/mL; ampicillin ≤ 2.0 µg/mL; linezolid ≤ 2.0 µg/mL; ciprofloxacin ≤ 0.5 - ≥ 8.0 µg/mL; vancomycin ≤ 1.0 µg/mL and teicoplanin ≤ 0.5 µg/mL.

Most of the clinical HLGR *E. faecalis* carried *cylA* gene; were recovered from blood (*n*=2) and abdominal puncture fluid (*n*=1). In food enterococci, *cylA* was detected in minced meat isolates (*n*=2) from ET4.

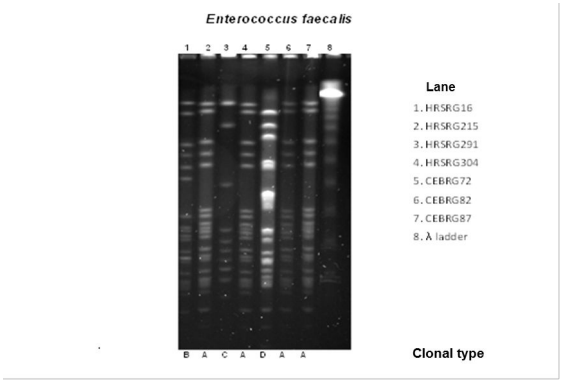

Figure 2: *Sma*I-PFGE patterns in high level gentamicin resistant *Enterococcus faecalis* isolates (Lane 1: *E. faecalis* HRSRG16 (human); Lane 2: *E. faecalis* HRSRG215 (human); Lane 3: *E. faecalis* HRSRG291 (human); Lane 4: *E. faecalis* HRSRG304 (human); Lane 5: *E. faecalis* CEBRG72 (food); Lane 6: *E. faecalis* CEBRG82 (food); Lane 7: *E. faecalis* CEBRG87 (food); Lane 8: lambda ladder).

*Sma*I-PFGE (Figure 2) showed that seven HLGR *E. faecalis* isolates were differentiated in four clonal types: EFA-A, EFA-B, EFA-C and EFA-D. Clone A was represented by four isolates, two from UCI´s patients HRSRG215 and HRSRG304 in the same period, isolated from blood and abdominal puncture fluid, respectively, and two from food, in minced meat from the same industrial plant (ET4) and period. Clones B (hemoculture), C (hemoculture) and D (handmade "salame") included one isolate each. Moreover, *cylA* gene was detected in pulsotypes EFA-A and EFA-B (Table 2).

Discussion

In the present study, *vanA E. faecium* was recovered from handmade "salame" and cheese. Previously, Barbosa et al. [40] did not detect vancomycin resistance in *E. faecium* isolated from traditional fermented meat products. Recently, Ribeiro et al. [41] found *vanA E. faecium* isolated from a traditional Portuguese dry fermented sausage. Therefore, the presence of *vanA E. faecium* is variable according to each analyzed geographic region.

Worldwide, VanA phenotype is linked to most of the human cases of vancomycin resistant enterococci, and is mainly carried by *E. faecium*. Spread of vancomycin-resistant *E. faecium* is a major global issue due to its persistence in hospital environment, limited therapeutic alternatives, and plasmidic *vanA* transfer [42].

Vancomycin-resistant *E. faecium* are the second most common cause of nosocomial infections in the US [43]. In Europe, vancomycin-resistant *E. faecium* prevalence is variable, ranging from less than 1% in France and Sweden to higher than 20% in Greece, Ireland, Portugal and the United Kingdom [44].

In Argentina, vancomycin-resistant *E. faecium* infections have been reported since 1998. In different hospitals predominance of one epidemic clone carrying *vanA* has been observed, with an increased incidence of vancomycin-resistant *E. faecium* clonal spread [24]. In 2014, according to the Antimicrobial Resistance Surveillance Network WHONET-Argentina (89 Microbiology Laboratories), clinical *E. faecium* shows a high rate (61%) of vancomycin resistance (www.antimicrobianos.com.ar).

In the current study, one pulso-type (VRE-B) was observed in two patients and in the mattress of another one, suggesting strain dissemination between patients and the environment. Kalocheretis et al. [45] detected vancomycin-resistant *E. faecium* transmission through a contaminated environment in different hospitals areas.

It should be noted that as a result of appearance of invasive infections at ICU-HMRS, surveillance of vancomycin-resistant *E. faecium* carriage in patients was started.

It is important to highlight that food vancomycin-resistant *E. faecium* isolates were susceptible to ampicillin and ciprofloxacin, but those of hospital origin were resistant to both antimicrobials. Ampicillin and, frequently, fluoroquinolone resistance are phenotypic markers for differentiating hospital and community vancomycin-resistant *E. faecium* [46].

In this research, only hospital *E. faecium* strains carried *esp* gene, also reported by Willems & Bontem [20]. According to Willems et al. [47], emergence of *E. faecium* ST-17 lineage in hospital settings started with adaptive changes, acquiring ampicillin resistance and a novel putative pathogenicity island, linked to *esp* gene. In CC17 strains, *esp* gene brings advantages in the adaptation to the hospital environment. Furthermore, the strong linkage between ampicillin and ciprofloxacin resistant *E. faecium* and CC17 has been widely accepted [48].

Isolate	Source	Sample	*cylA*/*esp*†	HLGR/VR*	*Sma*I-PFGE
E. faecium					
VREf-HRSER456	Clinical, ICU#††	Abdominal puncture fluid **	-/+	-/+	VREf-C
VREf-HRSER457	Clinical, ICU, ID	Hemoculture	-/+	-/+	VREf-C
VREf-HRSER458	Clinical, ICU, ID	Hemoculture	-/+	-/+	VREf-E
VREf-HRSER459	ICU. Environment	Mattress	-/+	-/+	VREf-B
VREf-HRSER460	Clinical, ICU, RC§	Rectal swab	-/+	-/+	VREf-B
VREf-HRSER461	Clinical, ICU, ID	Hemoculture	-/+	-/+	VREf-B
VREf-HRSER462	Clinical, ICU, ID	Rectal swab	-/-	-/+	VREf-G
VREf-HRSER501	Clinical, ICU, ID	Hemoculture	-/+	-/+	VREf-A
VREfHRSER503	Clinical, ICU. ID	Hemoculture	-/+	-/+	VREf-A
VREf-HRSER509	Clinical, RC	Rectal swab	-/+	-/+	VREf-A
VREf-CEBER95	Food, ET1§§	"salame"	-/+	-/+	VREf-D1
VREf-CEBER180	Food, ET3	Sheep cheese	-/+	-/+	VREf-D2
VREfCEBER616	Food, ET5	Cow cheese	-/+	-/+	VREf-F
E. faecalis					
EFA-HRSRG16	Clinical, ICU, ID	Hemoculture	+/-	+/-	EFA-B
EFA-HRSRG215	Clinical, ICU, ID	Hemoculture	+/-	+/-	EFA-A
EFA-HRSRG291	Clinical, ICU, ID	Hemoculture	-/-	+/-	EFA-C
EFA-HRSRG304	Clinical, ICU, ID	Abdominal puncture fluid	+/-	+/-	EFA-A
EFA-CEBRG72	Food, ET1	"salame"	-/-	+/-	EFA-D
EFA-CEBRG82	Food, ET4	Minced meat	+/-	+/-	EFA-A
EFA-CEBRG87	Food, ET4	Minced meat	+/-	+/-	EFA-A

†: cylA gene, esp gene; *HLGR: *aac(6')- Ie- aph(2")-Ia gene*; VR: *vanA* gene; ‡VREf: vancmoycin-resistant *E. faecium*; #ICU (Intensive Care Unit); ††ID: Invasive disease; ++: isolation date (mm/dd/yy); §RC: rectal colonization; §§ET: industrial plant; **EFA: high-level gentamicin resistant *E. faecalis*.

Table 2: Source and *Sma*I-PFGE of enterococcal isolates.

In this study, clonal complexes of *vanA E. faecium* were not investigated. Nevertheless, the presence of *esp* gene, ampicillin and ciprofloxacin resistance could suggest that clinical enterococcal isolates might belong to clonal complex 17, since its spread in Argentina has been reported (http://antimicrobianos.com.ar/2010/?cat=9).

The *vanA* gene is contained in Tn1546 or its derivatives, usually located on transferable plasmids. Plasmids are readily found in enterococci, and are common that clinical and commensal strains harbor a number of such elements. They comprise an important fraction of the auxiliary genome, and are responsible for much of the horizontal gene transfer that has allowed antibiotic and virulence traits to converge in hospital adapted lineages. Remarkably, different plasmid types occur in *E. faecium* and *E. faecalis*, despite their close phylogenetic relationship. The high content of plasmid and mobile genetic elements in *E. faecium* human lineages seems to be relevant to the emergence and persistence of their antibiotic resistance.

It is important to note that multiple drug resistance was not observed in food enterococci. Besides, it was not detected a clonal relationship between hospital-origin and food vancomycin-resistant *E. faecium*. Hammerum [17] considered that *E. faecium* isolates of animal origin are not themselves a hazard to humans, but they could act as donors of antimicrobial resistance genes to other pathogenic bacteria. The same variants of the *vanA* gene cluster, encoding for vancomycin resistance, have been observed in human and animal origin enterococci [49].

During 2013, in this study, in 10 patients with invasive infections, HLGR *E. faecalis* (40%) were detected. In Argentina, in 2014, according to WHONET Argentina, clinical HLGR *E. faecalis* (21.3%)

were recovered (http://antimicrobianos.com.ar/2016/01/informe-resistencia-2014-argentina/).

In this research, 3/14 (21.4%) food *E. faecalis* showed high-level gentamicin resistance. In Germany, Peters et al. [50] reported a low prevalence of HLGR enterococci isolated from food of animal origin.

In the current study, all characterized HLGR *E. faecalis* were not multi-resistant and showed a high prevalence of *cylA* gene. Additionally, *aac(6')- Ie- aph(2'')-Ia* gene was detected in all the isolates. Previously, in the same region, Sparo et al. [27] detected the spread of HLGR *E. faecalis* and this aminoglycoside resistance determinant in enterococci recovered from food, food producing animals and humans.

In human HLGR *E. faecalis*, different clonal types were observed (EFA-A, EFA-B, EFA-C). Previously, Murray et al. [51] found that several *E. faecalis* isolates with high-level gentamicin resistance from same and different locations presented distinct PFGE patterns. Few studies have shown the existence of clonal relationship between clinical isolates of *E. faecalis*. In this line, Larsen et al. [52] found HLGR *E. faecalis* ST16 with similar PFGE types, isolated from pigs, pork, non-hospitalized humans and patients with endocarditis. In the present research, the clonal type EFA-A isolates were distributed among food and human samples (minced meat, abdominal fluid and hemoculture). Food isolates were recovered from the same establishment (ET4) and processing period. These results showed the first evidence of clonal spread between human and food HLGR *E. faecalis* in Argentina. In Europe, Freitas et al. [53] observed the dissemination of a multidrug-resistant *E. faecalis* clone in animals and humans. Presence of transferable resistance mechanisms in food isolates constitutes a risk for humans through the food chain. Sparo et al. [2] proved *in vivo* horizontal transfer of high-level gentamicin resistance between intestinal microbiota and food *E. faecalis* strains.

Therefore, presence of mobile genes such as *vanA* in *E. faecium*, and *aac(6')- Ie- aph(2'')-Ia* in *E. faecalis* implies the need of a continuous sureveillance of these strains in hospitals and community settings [54].

Conclusion

In summary, in Argentina, there has not been so far evidence regarding the clonal relationship between food and human enterococcal strains. This is the first study that showed clonal relationship between *E. faecalis* with high-level gentamicin resistance isolated from food and humans. *vanA E. faecium* strains from hospital origin belong to different clonal types than *E. faecium* from animal food; although, it is important to emphasize that the number of isolates tested is small. Food *vanA E. faecium* were not identified as a direct cause of resistant enterococci in humans, but they could pose a risk for the intra or inter genus transfer of *vanA* resistance determinant to human-adapted strains.

Finally, this study demonstrated the spread of strains of *vanA E. faecium* and HLGR *E. faecalis* of different origin in the ecosystem, with potential for horizontal transfer resistance to other bacteria.

Author's Contributions

GP, MMDL and JB provided the samples for the study. GP, SL and CS were in charge of phenotypic characterization of isolates. AC and PG carried out molecular characterization. GD, SL and CS studied antimicrobial resistance. GD, JAB and MS analyzed results coordinated the different stages of the study. GP and JAB were responsible for an internal review of the study. GP, GD and MS were in charge of the writing of the manuscript.

Compliance with Ethical Standards

Ethical approval

Samples from human participants were obtained under Medical prescription. International and local legislation was followed. This article does not contain any studies with animals performed by any of the authors.

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