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Epidemiological and Molecular Investigation of Multidrug-Resistant *Enterococcus s*pp. Strains Isolated from Hospitalized Patients Using a One-Health Approach

Celia María Schell [a++*], Víctor Sebastián Blancato [b#], Ruth Jésica Carbone [c†], María Josefina Mauro [d‡], Vanina Torres [c†] and Christian Magni [b#]

[a] Centro Universitario de Estudios Microbiológicos y Parasitológicos (CUDEMyP), Centro Universidad Nacional de La Plata asociado a Comisión de Investigaciones Científicas (CIC), Facultad de Ciencias Médicas, La Plata, Argentina.
[b] Laboratorio de Fisiología y Genética de Bacterias Lácticas, Instituto de Biología Molecular y Celular (IBR-CONICET), Rosario, Santa Fe. Argentina.
[c] Laboratorio de Bacteriología. Hospital Interzonal General de Agudos Prof. Dr. Rodolfo Rossi. La Plata, Argentina.
[d] Hospital Interzonal General de Agudos San José de Pergamino, Buenos Aires, Argentina.

Authors' contributions

This work was carried out in collaboration among all authors All authors read and approved the final manuscript.

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[++] Professor and research;
[#] Professor and Head;
[†] Biochemistry;
[‡] Medical Doctor;
**Corresponding author: E-mail: schellcelia@med.unlp.edu.ar;*

ABSTRACT

Aims: The surveillance and investigation of antimicrobial resistance and virulence factor genes in nosocomial pathogens such as *Enterococcus spp.* have not been adequately prioritized within health institutions, especially in the context of COVID-19. The objective of this work was to conduct an epidemiological and molecular study in multidrug-resistant *Enterococcus spp*. strains isolated from hospitalized patients using a "One Health" approach in the middle of the COVID-19 pandemic.
Study Design: An observational, prospective, cross-sectional study was designed. All strains were isolated from hospitalized patients in a public hospital in La Plata, Buenos Aires, Argentina from September 2019 to August 2021; coincidental with the COVID-19 pandemic.
Methodology: In this study, we used N=17 *Enterococcus spp.* detected by biochemical testing and BD Phoenix™ M50: *E. faecalis* (n=6) and *E. faecium* (n=11). Antimicrobial susceptibility was determined according to standard guidelines (disk diffusion and MIC). AR and VF genes were detected by PCR assays.
Results: 10/11 (90.9%) vancomycin-resistant *E. faecium* strains were confirmed by molecular test. The highest detection of vanA-VREFM resulted after the pandemic cohort (2021). Resistance to glycopeptides was associated with resistance to ß-lactams and high-level resistance to aminoglycosides. The gen aac(6′)-Ie-aph(2″)-Ia was detected in *E. faecium* (n=7) and *E. faecalis* (n=1). The esp gene (52.9%) was the most frequent virulence factor, followed by gelE 7 (41.2%) and cylA 1 (5.9%) among *E. faecalis* and *E. faecium* isolates.
Conclusions: Nosocomial pathogens and commensal bacteria such as *Enterococcus* have an essential role in spreading AR and VF using genome plasticity to transfer genes located in MGE. The epidemiological and molecular investigation of multidrug-resistant strains allows adjusting biosafety protocols to prevent their spread.

Keywords: Antimicrobial resistance; Enterococcus faecalis; Enterococcus faecium; hospitalized patients; one health.

1. INTRODUCTION

Increasing bacterial drug resistance poses a serious threat to global public health in the coming years. The impact of unmonitored antimicrobial resistance (AR) is far-reaching and raises costs to patients, families, and health institutions [1]. The 68th World Health Assembly adopted an action plan under the "*One Health*" concept in 2015, to coordinate multisectoral efforts to prevent, prepare, and respond particularly focused on zoonotic diseases [1,2]. One of its strategic objectives is to intensify surveillance and research, especially in multidrug-resistant nosocomial pathogens [1]. In our country, the new law N° 27.680 of Prevention and Control of Antimicrobial Resistance was approved in 2022. However, the Coronavirus disease (COVID-19) pandemic complicated the rational use of antimicrobials due to the lack of proper worldwide-accepted protocols in many health institutions around the world and due to increase in risk of cross infection/nosocomial transmission coinciding with the increase in hospitalizations [3,4].

Vancomycin-resistant *Enterococcus faecium* (VREFM) is one of the most worrying problems in human health (CDC, 2022) [4]; together with *Enterococcus faecalis* both species are widely distributed in humans, animals, and the environment. Both contribute significantly to the increase in healthcare-associated infections (HAIs) and are relevant pathogens for immunosuppressed patients or with prolonged hospitalization [5,6]. They are considered one of the medical challenges of the XXI century due to the increasing prevalence of epidemic clones with multidrug-resistant (MDR), particularly those resistant to vancomycin and ampicillin [5-7]. High-risk clonal complexes (CC) CC2, CC9, and CC87 of *E. faecalis* are specially adapted to the hospital environment and widely distributed in Europe and America [5,7-9]. Penicillin-resistant and ampicillin-susceptible *E. faecalis* (PRASEF) have been reported since the late 1980s in different countries including Argentina and many of these strains produce β-lactamase [9-11].

The emergence of *E. faecium* strains belonging to the high-risk CC17 with associated resistance has become a global concern in recent years and have been found to belong to different clonal lineages associated with specific groups, with clonal lineage 17-BAPS 3.3a2 being one of the most concerning [7]. The clonal lineage 17-BAPS

3.3a2 comprises ST17, 18-BAPS 3.3a1, and 78-BAPS 2.1a. which have been associated with an increase in MDR *E. faecium* infections worldwide [7,8]. In Argentina, 74.3 % of *E. faecium* strains isolated from clinical samples were resistant to vancomycin and belonged to CC17, according to the National Surveillance of Antimicrobial Resistance (partial data for 2010-2021 - WHONET Network) [12].

E. faecium and *E. faecalis* have acquired resistance to vancomycin, ampicillin, and exhibit high-level resistance to aminoglycosides (HLRA). *E. faecium* acquires resistance to β-lactams mediated by two mechanisms: generation of the low-affinity class B penicillin-binding protein (particularly PBP5) in *E. faecalis* sometimes denominated PBP4, and β-lactamase production [13,14]. The AR to glycopeptides is achieved by target modification by eliminating the high-affinity peptidoglycan precursors throughout enzymes present in the *van*-operons [12,14]. The most frequent *van* genotypes found in *Enterococcus* are *vanA* (located in Tn*1546*) and *van*B (Tn*1549* and Tn*5382*) [14].

A high level of gentamicin (MIC ≥ 500 µg/mL) and streptomycin (MIC ≥ 2000 µg/mL) resistance was reported as early as 1970 [15]. This mechanism is mainly due to the presence of the bifunctional aminoglycoside-modifying enzyme, AAC(6')-APH(2”), which can be found in transposons or plasmids. More than 90% of clinical isolates of *Enterococcus* possess *aac(6')-Ie-aph(2'')-Ia* gene that codes for this enzyme [14,15].

The relevance of the *Enterococcus* genus lies in its ability to incorporate, transmit, and disseminate mobile genetic elements (MGE) through horizontal gene transfer (HGT) [16-18]. By inhabiting various ecological niches, both suitable and adverse (high pH and inanimate reservoirs), and through genetic evolution (genetic capitalism), *Enterococcus* can improve its genome.

Thus, *Enterococcus* expresses virulence factors (VF) and AR, primarily to glycopeptides, aminoglycosides, and β-lactams [17,19,20]. Some of the most important VF in *Enterococcus* include the production of enterococcal surface protein (Esp), gelatinase (GelE), and hemolysin-cytolysin (CylA). Understanding the pathogenicity of enterococcal infections is improved by studying these VF as they contribute to the severity of enterococcal infections by enhancing colonization and invasion [19,21]. In this scenario, the eventual resolution of the infection (e.g., bacteraemia, endocarditis, abscess, meningitis) is often complicated.

The objective of this work was to conduct an epidemiological and molecular study in MDR *Enterococcus* spp. strains isolated from hospitalized patients using a "*One Health*" approach in the middle of the COVID-19 pandemic.

2. MATERIALS AND METHODS

2.1 *Enterococcus* spp. Strains: Isolation and Identification

An observational, prospective, cross-sectional study was designed. *Enterococcus* spp. strains were recovered and isolated from different clinical sources of patients with clinically documented infections who were hospitalized at the Interzonal General Acute Hospital (I.G.A.H.) Prof. Dr. Rodolfo Rossi (La Plata, Buenos Aires, Argentina) between September 2019 and August 2021. This institution is a high-complexity center and belongs to the XI sanitary region of the Province of Buenos Aires, Argentina. The population consisted primarily of adult patients between 35 to 64 years old (56%), and 2% of the assisted population was from neighboring countries (https://www.ms.gba.gov.ar/sitios/hrossi/).

Enterococcus strains (one colony per morphology per patient) were isolated from blood, urine, bone samples, and abscesses principally. All strains were identified with conventional biochemical tests [22] and confirmed by BD Phoenix™ M50. Note: Some epidemiological data could not be obtained during the study, because Electronic Medical Records were not implemented yet.

The strains were stored in University Center for Microbiological and Parasitological Studies (CUDEMyP-UNLP-CIC) at -70ºC on Brain Heart Infusion (Britania Laboratory) + glycerol 20%. Molecular analysis was performed on CUDEMyP-UNLP-CIC and Institute of Molecular and Cellular Biology (IBR-CONICET), Rosario, Santa Fe, Argentina.

2.2 Antimicrobial Susceptibility Test

The phenotypic profile of AR was determined using the disc diffusion method and the

automated BD Phoenix™ M50 system, in accordance with the guidelines set by the Clinical and Laboratory Standards Institute [23]. Susceptibility to 10 antibiotics was evaluated, including ampicillin (10 µg), ampicillin/sulbactam (20 µg), vancomycin (5 µg), teicoplanin (30 µg), amikacin (30 µg), streptomycin(300 µg-high loads), gentamicin (120 µg-high loads), tetracycline (30 µg), ciprofloxacin (5 µg), and linezolid (30 µg).

The minimal inhibitory concentration (MIC, µg/mL) was detected using the epsilometric method (E-test) and BD Phoenix™ M50 system (daptomycin, gentamicin, streptomycin, linezolid, vancomycin, teicoplanin, ampicillin). Daptomycin only MIC, for those who have automated or epsilometric method, because the diffusion method is not validated (ANLIS/INEI/PT-WHONET-ARG; 2022). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as control strains.

2.3 β-Lactamase Production

Was realized by the nitrocefin test (BD BBL, Franklin Lakes, NJ, USA), according to the manufacturer's instructions [11,24]. *S. aureus* ATCC 29213 was used as a positive control.

2.4 Efficient DNA Extraction and Detection of Antimicrobial Resistance and Virulence Genes

To extract genomic DNA from fresh colonies, the boiling method (100 °C for 15 minutes) was employed as previously described [25]. DNA concentration (ng/µL) was determined by measuring the absorbance at 260 nm using a microplate reader (Bio-Tex). DNA purity was assessed by calculating the 260/280 ratio.

PCR assays were carried out using standardized protocols to detect the presence of *vanA*, *vanB*, *vanC-1*, and *vanC-2* genes (which confer resistance to glycopeptides) [26] as well as *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id* genes (which confer resistance to aminoglycosides) [27,28]. Positive and negative controls were included for each gene in the assay, which was performed on a Veriti Thermal Cycler (ThermoFisher Scientific). The *esp*, *gelE,* and *cylA* genes were detected as previously described by Pourcel *et al*., (2017) and Eaton & Gasson (2001) [25,29].

PCR was performed in a final volume of 15 µL using 0.6-1.6 ng of bacterial DNA (depending on the sample), 0.4 µM of the specific primers, 1 U of Taq DNA polymerase, 50 µM of each dNTP, 1.5 mM $MgCl_2$ in a buffer solution containing TrisHCl 10 mM pH 8, KCl 50 mM. The PCR products were separated by electrophoresis (Amersham Biosciences) in 1.5% agarose gels with TAE (Tris/Acetate/EDTA) buffer 1X, at 100 V, and 300w for 30 min. The gels were stained with SYBR Safe (Invitrogen-Thermo -Fisher Scientific) and visualized under blue light illumination. Lambda DNA digested with EcoRI+HindIII enzymes was usedas a molecular weight marker. The primer sequences used for identifying AR genes and virulence determinants by PCR can be found in Table 2.

2.5 Hemolysin Production

The hemolytic ability was assessed phenotypically on an Agar base supplemented with 5% sheep blood (Argentine Laboratory) and fresh human blood. After 24 hours at 35-37°C, the formation of clear zones around bacterial colonies indicated β-hemolysis. Greenish zones around colonies were characteristic of α-hemolysis, while the absence of any zones indicated γ-hemolysis.

2.6 Heat Map

Binary coding was used to represent the antibacterial susceptibility profile, the existence of drug-resistant genes, and VF. "0" denoted sensitivity for each antibiotic under test, while "1" denoted resistance. Similar to this, a gene or VF existence was denoted by a "1" and its absence by "0". The heatmaply for the R programming language was then used to produce a distance -based clustering heatmap [30]. The strain and isolation year were both indicated on this heatmap.

2.7 Statistical Analysis

Was performed using specialized software. Descriptive statistics (Chi-square (X^2) test**)** were used to present the results in terms of relative frequency. A significance level of $P < 0.05$ was established to determine statistical significance.

Table 1. Relevant phenotypic and genotypic characteristics of *Enterococus* isolated in I.G.A.H., La Plata. Period: 2019-2021

Strain	Year of isolation	Species	Antibiotic Resistance profile	VRE* (genotype)	*aac(6')-Ie-aph(2'')-Ia*	Virulence gene	Product of a HAIs
HR21/3652	2021	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	*esp*	No
HR21/3612	2021	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK, TCY	*vanA*	+	ND	+
HR21/2756	2021	*E. faecalis*	CIP, GEH, AMK	-	+	*gel*	No
HR21/3642	2021	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	ND	+
HR21/3661	2021	*E. faecalis*	AMK	-	ND	*gel, cylA*	+
HR21/1802	2021	*E. faecium*	CIP, AMP, SAM, AMK	-	ND	*gel*	No
HR21/1798	2021	*E. faecium*	VAN, TEC, CIP, AMP, SAM	*vanA*	ND	*esp*	No
HR21/1733	2021	*E. faecalis*	AMK	-	ND	*gel*	No
HR21/1685	2021	*E. faecalis*	AMK	-	ND	*gel*	No
HR21/1676	2021	*E. faecium*	LZN, VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	*esp, gel*	No
HR057/20	2020	*E. faecalis*	AMK	-	ND	*esp, gel*	No
HR368/20	2020	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	*esp*	+
HR002/20	2020	*E. faecium*	VAN, TEC, CIP, AMP, SAM, AMK	*vanA*	ND	*esp*	No
HR6344/19	2019	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	*esp*	No
HR5254/19	2019	*E. faecium*	VAN, TEC, AMP, SAM, AMK	*vanA*	ND	*esp*	+
HR5172/19	2019	*E. faecium*	VAN, TEC, CIP, AMP, SAM, GEH, AMK	*vanA*	+	*esp*	No
HR4808/19	2019	*E. faecalis*	AMK	-	ND	ND	+

Table 2. Primer sequence (5'– 3') of antimicrobial resistance and virulence determinant genes used to *Enterococcus* spp. isolated from clinical samples from hospitalized patients. Period: 2019-2021

Gene	Primer sequences (5'–3')	Annealing temperature (°C)	Amplicon size
vanA	F: *GGGAAAACGACAATTGC* R: *GTACAATGCGGCCGTTA*	54 °C	732 bp
vanB	F: ACGGAATGGGAAGCCGA R: *TGCACCCGATTTCGTTC*	54 °C	647 bp
vanC1/2	F: *ATGGATTGGTAYTKGTAT*[c] R: *TAGCGGGAGTGMCYMGTAA*[c]	54 °C	815/827 bp
aac(6')-Ie-aph(2")-Ia	F: *CAGGAATTTATCGAAAATGGTAGAAAAG* R: *CACAATCGACTAAAGAGTACCAATC*	54 °C	369 bp
aph (2´´)-Ib	F: CTTGGACGCTGAGATATATGAGCAC R: GTTTGTAGCAATTCAGAAACACCCTT	54 °C	867 bp
aph (2´´)-Ic	F: CCACAATGATAATGACTCAGTTCCC R: GCCACAGCTTCCGATAGCAAGAG	54 °C	444 bp
aph (2´´)-Id	F: GGTGGTTTTTACAGGAATGCCATC R: CCCTCTTCATACCAATCCATATAACC	54 °C	641 bp
esp	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	62 °C	933 bp
gelE	F: TATGACAATGCTTTTTGGGAT R: AGATGCACCCGAAATAATATA	56 °C	213 bp
cylA	F: TGGATGATAGTGATAGGAAGT R: TCTACAGTAAATCTTTCGTCA	52 °C	517 bp

[c]: K = G or T; M = A or C; Y = C or T

3. RESULTS AND DISCUSSION

3.1 *Enterococcus* spp. Isolated from Hospitalized Patients of I.G.A.H. Prof. Dr. Rodolfo Rossi (La Plata, Buenos Aires, Argentina)

A total of 17 enterococcal strains were isolated and selected from clinical samples of hospitalized patients, including those from the bone marrow transplant unit, between September 2019 and August 2021. Of these, 11 (64.7%) were identified as *E. faecium*, and 6 (35.3%) as *E. faecalis*. Table 1 shows the strains data. The highest number of isolates was observed in the post-pandemic cohort (*n*=10 *Enterococcus* spp. in 2021). The frequency percentage of HAIs-producing strains was 35.3% and 64.7% for non-producers. No statistically significant association was demonstrated between isolated species and HAIs production (*P*=0.901).

3.2 Antimicrobial Susceptibility and Molecular Testing Reveal MDR *Enterococcus* and Genotype Circulation

Table 1 summarizes the relevant phenotypic and genotypic characteristics, of the 17 strains, 10 (58.8%) were identified as VREFM and exhibited an MDR profile. Resistance to glycopeptides (100% to vancomycin and teicoplanin) in VREFM was associated with resistance to beta-lactams: ampicillin (100%), ampicillin/sulbactam (100%), high-level gentamicin resistance (70%) and amikacin (90%). Resistance to ciprofloxacin (90%), and linezolid (10%) were detected in VREFM too.

In contrast, *E. faecalis* isolates (*n*=6) were generally susceptible to all antibiotics tested, except for ciprofloxacin (20%), amikacin (100%) and gentamicin (20%) at a low percentage.

The MIC of gentamicin was ≥ 500 µg/mL in all strains that demonstrated resistance to this antibiotic. The MIC of vancomycin was > 32 µg/mL equal to teicoplanin; ciprofloxacin > 8 µg/mL and ampicillin > 32 µg/mL. The MICs range of daptomycin was ≥ 1-2 µg/mL (CLSI, 2023) in all strains.

The *vanA* genotype was detected in 10 VREFM strains (90.9%) with a statistically significant association (*P*=0.00). The *vanB, vanC1,* and *vanC2* genes were not detected in this study (Table 1). Furthermore, there was no observed production of β-lactamase.

3.3 High Prevalence of Aac(6') -Ie-Aph(2'')-Ia (Aminoglycoside-Modifying Enzyme Gene) Among Isolates of *E. faecium*

The *gen aac(6')-Ie-aph(2")-Ia* (369 bp) was found in *E. faecium* (*n*=7) and *E. faecalis* (*n*=1) (Photography 1). The *aac(6')-Ie-aph(2")-Ia* gene was detected in all isolates exhibiting high levels of resistance to gentamicin, however no statistically significant association between species was observed (*P*=0.064). This finding was consistent with MIC GEH > 500 µg/mL and MIC STH < 2000 µg/mL. On the other hand, the *aph(2´´)-Ib* (867 bp), *aph(2´´)-Ic* (444 bp), and *aph(2´´)-Id* (641 bp) genes were not detected in this study (Table 1).

Photography 1. Representative image of the amplified PCR products of high level resistance to gentamicin gene: *aac(6')-Ie aph(2")-Ia* of *Enterococcus* isolates (expected size: 369 bp). Lane 10: molecular weight marker

3.4 Virulence Profiles of the *Enterococcus spp.* Isolates and Hemolytic Activity

Among the 17 *Enterococcus* spp. clinical isolates, 15 (88.2%) were found to possess virulence genes (Table 1). The *esp* gene was the most frequently detected (58.8%), followed by *gelE* (41.2%) and *cylA* (5.9%) among the *E. faecalis* and *E. faecium* isolates. Statistically significant association was observed with *gelE* (*P*=0.009) and *esp* (P=0.027) between species.

Three isolates exhibited an association of two virulence genes:

- *gelE* + *cylA* in the HR21/3661 *E. faecalis* strain isolated from a catheter-tip clinical sample of a deceased female inpatient from the intensive care unit (ICU) with hospital-acquired pneumonia.
- *gelE* + *esp* in the HR057/20 *E. faecalis* strain isolated of bone infection (osteomyelitis).
- *gelE* + *esp* in the HR21/1676 *E. faecium* strain (no epidemiological data).

None of the *Enterococcus* strains isolated in this study exhibited β-hemolysis. However, some isolated strains exhibited α-hemolysis (52.9%) and γ-hemolysis (47.1%).

3.5 Heat Map Displaying Antibiotic-Resistant *Enterococcus* Isolates Illustrates the Clustering Based on their Distances

The Fig. 1 shows a comparative presence of resistance genes and VF reveals two major clusters where *E. faecalis* separated from *E. faecium* strains, except for *E. faecium* H21/1802 which is closer to *E. faecalis* group. Binary factors are represented by blue for presence (with a relative response of 1) and light blue for absence (with a relative response of 0). *E. faecium* cluster showed much more resistance and virulence genes than *E. faecalis*. The year of isolation of the samples does not seem to be correlated with the clusterization.

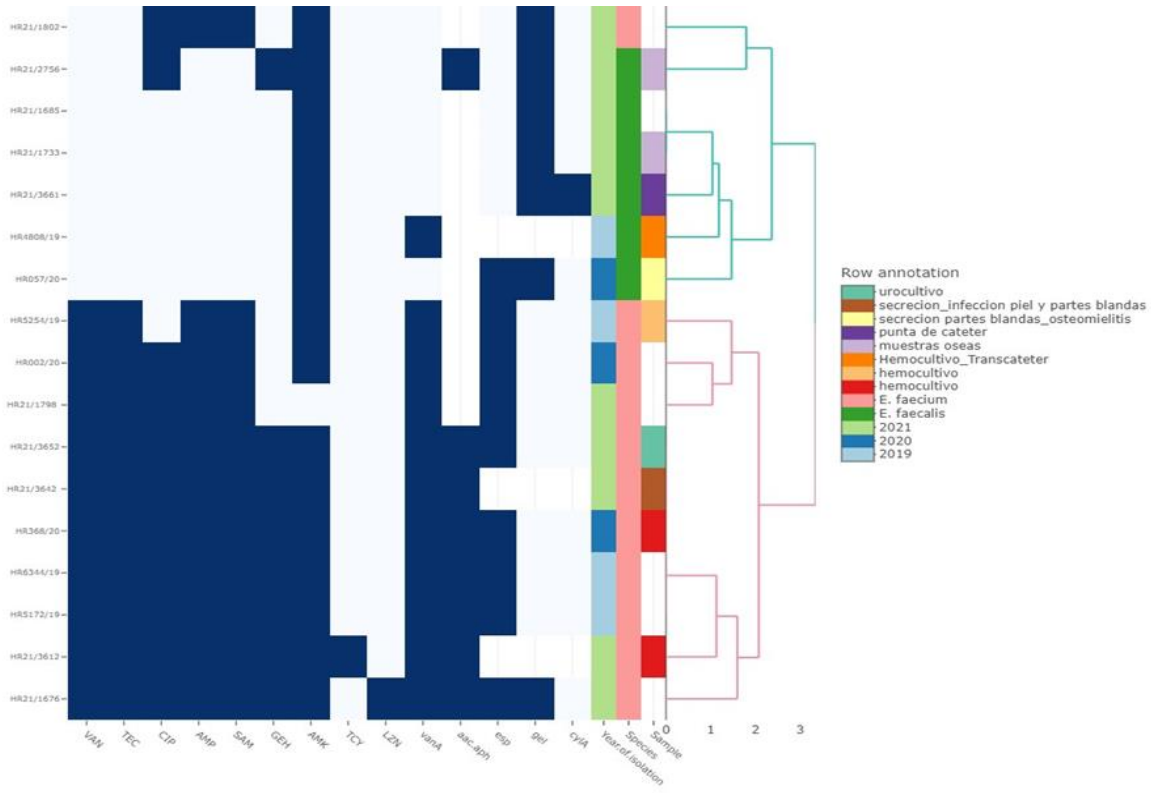

Fig. 1. Heat map displaying antibiotic-resistant and VF in *Enterococcus* isolates illustrates the clustering based on their distances

4. DISCUSSION

When COVID-19 began (on March 11, 2020, The WHO declared it a pandemic), the world's health systems gradually began to collapse [3,31]. Resources for public health, such as diagnoses of other pathologies, distribution of medicines (including cancer drugs and antimicrobials), and treatments for chronic patients, were also affected and delayed [31-33]. In this adverse context, many patients were admitted to the ICU due to infection with Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), where morbidity and mortality rates were increased, compounded by the susceptibility of the patient to HAIs [34]. The genus *Enterococcus* is part of the human and animal gut microbiota. However, it colonizes inanimate reservoirs of intra-hospital ecological niches.

In addition to the COVID-19 pandemic, AR poses a significant threat to the healthcare system. The CDC's 2022 Antibiotic Resistance Threats Report highlights the importance of prevention in protecting people from antimicrobial-resistant infections and their spread. AR is a leading cause of death globally, with the highest burden in low resource countries [35,36].

In this study, we conducted the first epidemiological surveillance of *Enterococcus* spp. isolated from an inter-zonal public hospital. Strains were isolated from clinical samples of hospitalized patient covering the period from September 2019 to August 2021 and including those from the bone marrow transplant and COVID units. Due to the complications arising from the pandemic in terms of biosafety standards and strain collection and transfer, we selected only the most clinically relevant strains of *Enterococcus* for analysis. A total of 11 *E. faecium* and 6 *E. faecalis*, were identified. Mainly, the study focused on VREFM. Despite the limited sample size (was small due to Electronic Medical Records were not implemented yet), observed a higher prevalence of VREFM (58.8%). These results are consistent with the findings of Fukushige *et al*. [33], who reported HAIs due to VREFM at a teaching hospital in southern Taiwan during the COVID-19 era. Similarly, Kampmeier *et al*. (2020) demonstrated the transmission of vancomycin-resistant *Enterococcus* (VRE) in an ICU using whole genome sequencing (WGS) during the ongoing COVID-19 pandemic, detecting the presence of VREFM in clinical and environmental samples [37]. *Enterococcus*, especially VRE, are known to survive on hospital environmental surfaces in dry conditions, such as medical equipment and inanimate surfaces, and their nosocomial transmission is frequent, particularly among patients with multiple comorbidities admitted to the ICU [38,39]. The frequency of *Enterococcus* found in our study differs from the results reported by Schell *et al*. (2020), who found a higher prevalence of *E. faecalis* than *E. faecium* in invasive infections in a public hospital of Tandil, Buenos Aires, Argentina [9]. Understanding the epidemiology of bacteria carrying resistance mechanisms located in quickly disseminated MGE within healthcare institutions is crucial. Therefore, there is a need to strengthen measures to detect and prevent their transmission.

In this hospital, the major percentage of resistance was observed against gentamicin (41.2%), vancomycin (58.8%), teicoplanin (58.8%), ampicillin (58.8%), ampicillin/sulbactam (58.8%), and ciprofloxacin (52.9%) in VREFM. However, *E. faecalis* only exhibited resistance to ciprofloxacin and gentamicin in a low percentage. These findings are consistent with data from the Latin American Surveillance of Antimicrobial Resistance (ReLAVRA) and RED-WHONET in 2018 and 2021, which analyzed *E. faecalis* and VREFM isolated from HAIs in Argentina. The report also indicates that the prevalence of MDR *E. faecium* increased relative to the isolation of *E. faecalis* in our country (National-Surveillance-of-Antimicrobial-Resistance-Red-WHONET-Argentina-Trend-2010-2021.pdf).Conversely, Jovanović *et al*., (2023) reported the isolation of vancomycin-susceptible invasive enterococcal strains (14 *E. faecalis*, 2 *E. faecium*, and 1 *E. durans*) obtained from blood cultures of patients from three clinics in Belgrade, Serbia (to January 2017-December 2019) [40]. Although these findings provide valuable insights, further research is necessary to gather epidemiological data on the AR profile of various healthcare institutions. This will enable the identification of appropriate drugs to enhance empirical therapy and to intervene in the control and prevention of AR in the geographic region. The continuous collection of reliable, comparable, and reproducible data on AR profiles is critical for effective intervention.

This study discovered a higher occurrence of the *vanA* genotype in 10 out of 11 *E. faecium* strains aligns with findings reported by Corso *et al*. (2007) in Argentina and Saengsuwan *et al*. (2021) in Thailand [12,41]. In our country, the

vanA is the most prevalent circulating genotype in *E. faecium*. Both *vanA* and *vanB* are in MGE. Given the rise in VRE infections among hospitalized patients, controlling the prevalence of VRE in healthcare facilities has become a major concern for both public health experts and our hospital.

To keep the active surveillance of the epidemiological profile updated and thus prevent therapeutic failures, the prevalence of strains with reduced susceptibility to daptomycin was evaluated. no daptomycin-resistant VREFM strains were observed (MIC: 2 - ≥ 1 µg/mL) in this study. Since the clonal dissemination of certain strains in some health institutions may be overestimated and there may be an emergency to AR to daptomycin, strict monitoring of the susceptibility and resistance patterns of these microorganisms is necessary [42].

The frequency of HLRA genes was found to be 47%. Among the detected genes, only the *aac(6')-Ie-aph(2")-Ia* was identified: 7 *E. faecium* and 1 *E. faecalis*. This gene is considered the most clinically significant among these genes. It encodes the bifunctional enzyme AAC (6')-APH (2"), and eliminates the synergistic bactericidal effect typically observed when combining a cell wall-active agent with an aminoglycoside [19,43]. These results are consistent with the results conducted in 2018 by Amini *et al*., who reported that 42.6% of their *Enterococcus* were positive for *aac(6')-Ie-aph(2")-Ia*. However, these findings do not align with published by Sparo *et al*., (2013), who reported the detection of the *aac(6')-Ie-aph(2")-Ia* gene in all *E. faecalis* isolated from blood culture (11/11) [28].

aph(2´´)-Ib, *aph(2´´)-Ic*, and *aph(2´´)-Id* genes were not detected in this study, coinciding with Moussa *et al*., (2019) who did not find *aph(2")-Ib, aph(2")-Ic* and *aph(2")-Id* among the study isolates [44]. Cercenado (2011) expresses that more than 90% of the clinical isolates of *Enterococcus* that present AR to gentamicin have *aac(6')-Ie-aph(2")-Ia* gene and less than 10% have the genes *aph(2")-Ib, aph(2")-Ic*, *aph(2")-Id* [14]. These genes have been reported to be either integrated within MGE or encoded on plasmids. Due to the rapid dissemination of this genetic determinant, the impact of its horizontal transferability among enterococcal species from various sources must be considered.

In terms of VF detection, 82.35% of the strains were found to possess VF (Table 1). The *esp* was the most frequently detected virulence gene (52.9%), followed by *gelE* (41.2%) and *cylA* (5.9%) among the *E. faecalis* and *E. faecium* isolates. In *E. faecalis*, Esp contributes to colonization and biofilm formation. However, the presence of the variant *esp* gene in VREFM appears to be associated with increased virulence [45,46]. Interestingly, we observed a higher proportion of VREFM strains (*n*=8) with the *esp* gene compared to *E. faecalis* strains (*n*=1). Leavis *et al*., (2004) demonstrated the presence of a novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *E. faecium*, which coincides with the nosocomial outbreaks of *E. faecium* during our study, however, for its confirmation, comparative genomics studies could be carried out [47]. In VREFM, the frequency of the gene encoding Esp has been found to be significantly higher among clinical isolates recovered from infected patients and hospital outbreaks, particularly in relation to epidemic-virulent CC-17 strains of *E. faecium* [48-50].

Gelatinase is a zinc metalloprotease with hydrolytic capacity encoded by the *gelE* gene [51]. As a result of the enzymatic hydrolysis of fibrinogen the bacterial migration into host tissue could be enabled because of the disruption of enterocytes [51]. Further, the tissue degradation of the host provides nutrients to bacteria and contributes to the biofilm formation process. In our study, a higher percentage of *gelE* detection was observed in *E. faecalis* (29.4%) than *E. faecium* (11.7%). The role in the pathogenesis of *E. faecalis* is not clear, although many studies communicate the presence of *gelE* in clinical strains [52,53]. Arciola *et al*., (2008) conducted an analysis on a collection of isolates of *E. faecalis* obtained from orthopedic implant infections [54]. The study revealed that a significant proportion of *gelE*-positive strains exhibited a robust expression of the gene and an MDR, particularly among epidemic clones. Our results are in concordance with Kiruthiga *et al*., (2020) who documented that *gelE* was the second most common (76.4%) gene detected in *Enterococcus*, more commonly in *E. faecalis* (85.39%) than *E. faecium* (60.78%) [54].

Cytolysin is a hemolytic toxin that occurs in up to 60% of *E. faecalis* strains. The operon is located either on a plasmid or integrated into the bacterial chromosome, with the most studied being pAD1 [55]. Causes the lysis of red blood cells (β-hemolytic activity). *E. faecalis* strains expressing *cyl* are more virulent than those

isogenic strains that do not express it [55,56]. In this study, the *cylA* gene was detected in only one strain of *E. faecalis*, different results were published by Sparo *et al*., (2013) who demonstrated the presence of *cylA* in all strains of this species (*n*=11) isolated from blood cultures [28]. The inherent capacity of *Enterococcus* to easily acquire, accumulate, and exchange extrachromosomal elements that carry VF or AR genes provides them with advantages for surviving in atypical environmental conditions with unique ecological conditions [57]. This, in part, elucidates their growing significance as nosocomial pathogens.

This project was affected by the COVID-19 pandemic, resulting in several limitations. However, this study represents the initial report on the molecular investigation of AR and VF in a sample of *E. faecalis* and *E. faecium* isolated from hospitalized patients, using a comprehensive holistic *One Health* approach. By integrating professionals from the School of Medicine-UNLP, Institute-IBR, and the Hospital, this collaborative, interdisciplinary effort examined resistance patterns and specific VF within the local context and shows the presence of *vanA*-VREFM strains circulating in the hospital, especially after the start of the pandemic (2021). Additionally, it explored strains carrying MGE that contribute to the dissemination of these traits within the intrahospital microbial community.

In this hospital as in others, the elevation of AR mechanisms in pathogens such as *Enterococcus*, was multifactorial. For example: indiscriminate use of antimicrobials when complications occurred in patients with COVID, requiring longer days of hospitalization. In outpatients, with pathologies such as diabetic foot where *Enterococcus* are isolated, it was observed that the pandemic complicated periodic controls in these patients. It is necessary to carry out an in-depth epidemiological study to confirm these observations using a larger sample number.

5. CONCLUSIONS AND SUGGESTIONS

This is the first study of *Enterococcus* in a public hospital under the “One Health approach” and in the context of a COVID-19 pandemic.

Understanding the characteristics of the resistome in each healthcare institution enables targeted interventions and strategies. In cases where pathogens that pose challenges to the efficacy of frontline antibiotics are identified, alternative approaches become imperative. Nosocomial pathogens and commensal bacteria such as *Enterococcus* have an essential role in spreading AR and VF using genome plasticity to transfer genes located in MGE. This project focused on implementing specific objectives outlined in the Prevention and Control of Antimicrobial Resistance (New Law N° 27.680; https://www.boletinoficial.gob.ar/detalleAviso/primera/270118/20220824) in Argentina.

Although these findings provide valuable insights, further research is necessary to gather epidemiological data on the AR profile of various healthcare institutions. This will enable the identification of appropriate drugs to enhance empirical therapy and to intervene in the control and prevention of AR in the geographic region. The continuous collection of reliable, comparable, and reproducible data on AR profiles is critical for effective intervention.
Further, in necessary to adjust and comply with the biosecurity protocols to contain the spread of nosocomial pathogens is crucial in reducing HAIs.

Since the clonal dissemination of certain strains in some health institutions may be overestimated and there may be an emergency to AR to daptomycin, strict monitoring of the susceptibility and resistance patterns to daptomycin in these microorganisms.

6. LIMITATIONS

We acknowledge two limitations of the study. First, the sample size was small due to the lack of epidemiological data on the patients during that period. The hospital did not have an electronic record system at that time, which limited the availability of data. Second, the COVID-19 pandemic has had a significant impact on transportation and logistics worldwide, making it difficult to collect and transport samples safely.

AVAILABILITY OF DATA AND MATERIALS

All the data supporting the conclusions of this article is included within the article.

CONSENT AND ETHICS APPROVAL

Patient records were obtained in accordance with National Law Nº. 25.326, Article 11 of "Personal

Data Protection," and the National Law Nº 26529/10 "Patient Rights, Clinical History and Informed Consent" of Argentina, which are in line with the Helsinki statement. This project was evaluated and approved by the Committee on Bioethics and Research Ethics (COBIMED), Accreditation Nº 047/2014, of the School of Medicine at the National University of La Plata and authorized by the health institution. An internal alphanumeric code was assigned to each isolated strain to maintain confidentiality.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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