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Investigation of the epidemiology and antibiotic resistance of multi-drug resistant enterococcal and staphylococcal strains isolated from clinical samples

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Abstract

The increase of vancomycin-resistance due to co-colonization or co-infection with Enterococci (VRE) and Staphylococci (MRSA) in Algerian hospital settings is poorly reported, in which, few data of the molecular mechanism have been accessible on study of this resistance across the country. In this study, we investigate the epidemiology and antibiotic resistance of multi-drug resistant enterococcal and staphylococcal strains isolated from various clinical samples taken from hospitalised patients in Biskra hospital and outpatients within the study period from 2019 to 2021. All strains collected were identified using VITEK® 2 system, MRSA and VRE phenotypic confirmation by vancomycin and teicoplanin E-test were carried out.

A total of 24,642 patients were analysed, of which 270 patients harboured staphylococcus including 80 MRSA and 135 Enterococci including 37 VRE. We identified 37 case patients for whom a single clinical specimen yielded both VRE and MRSA on culture. We carried out univariate comparisons between the 2 groups, then a multivariate logistic regression analysis. Results revealed that *Staphylococcus aureus,E. faecium and E. faecalis* strains are the main pathogens identified in this study. The majority of strains were detected in intensive care unit (ICU) (56.8%) followed by surgical wards (SW) 29.70%. The highest prevalence was found on pus (67,5%) followed by the blood culture (32.6%)The rate 52.2% of patients harboring both (MRSA) and (VRE) was male. Antibiotic susceptibility testing revealed that the resistance rate was high for the majority of antibiotic classes, including glycopeptides in which, MIC VAN > 256 µ g/mL and MIC TEC > 256 µ g/mL was observed for all resistant strains.

Introduction

Antibiotic resistance has become more widespread worldwide. Hospitals are high-risk areas for transmission of serious nosocomial infections especially affected by highly resistant pathogens. Nosocomial infections due to Gram positive bacteria rank among the top leading causes of death and

increased morbidity among hospitalized patients. Various Gram positive bacteria have been identified as multi-drug resistant pathogens, but the major two genders are Staphylococcus and *Enterococcus* which cause many important human diseases as bacteraemia, endocarditis, urinary tract infections, surgical wound infections, intra-abdominal and intra-pelvic infections. The most worrying risk factor is co-colonization by these two bacterial strains, causing a therapeutic impasse.(5)

Nowadays, Staphylococcus remains the most frequently isolated and pathogenic Gram-positive bacteria responsible for multiple infections in hospitals [1]. The most dangerous strains are those that are multi-resistant to antibiotics. This is the case of MRSA (*methicillin-resistant staphylococcus*) which poses a major public health problem [2].

In other hand, *Enterococcus* belongs to the group of Gram-positive bacteria that are regarded normal colonizers of the human gastrointestinal tract, but of great relevance to human health for their role as major causative agents of health care-associated human's infections. The greatest threat these bacteria represent is their ability to developed multi-resistance to virtually all antimicrobials currently used and to transfer resistance genes to *Staphylococcus aureus.* Consequently, they can cause serious invasive diseases (3), by the acquired of antibiotic resistance which limits the number of treatment approach (4). This intrinsic antimicrobial resistance played a role in providing opportunities for enterococci to interact with other drug-resistant bacteria, by acquiring or transferring additional resistances on mobile elements

Indeed, several reports have suggested that the near existence of VRE and MRSA may be one of the risk factors in the transfer of drug-resistant genes and development of antibiotic resistance [6]. In that context, many surveys investigated the epidemiology and antimicrobial susceptibility of VRE and MRSA concurrently, isolated from the different human specimens.

Over the last two decades the high mortality rate caused by these dangerous strains is alarming because of their increasing of multiple antibiotic resistances, which is being a serious threat to current healthcare practices. Despite its importance, few publications have been made concerning this study in Algeria and our survey is considered the first to estimate the prevalence and risk factors of patients who are co-colonized by VRE and MRSA.

The main aim of this study was to determine the epidemiology and antibiotic resistance of multi-drug resistant enterococcal and staphylococcal strains isolated from clinical samples.

Materials & Methods

This prospective study was conducted in the microbiology laboratory of SAADANE General Hospital (public hospital institution) in southeastern Algeria, for period of 12 months (August 2019 to August 2020).Our isolates were collected from different pathological origins of community infections and clinical samples (blood, urine, pus, and throat swabs, wound and ear swabs .uro-genital fluids).

Concerning the collection of staphylococcus strains, our study was interested in the strains which showed resistance to Methicillin (SMR) or those multi drugs. All strain of staphylococci was selected by growing on agar Chapman to ovoid contamination and Colombia blood agar media, then incubating aerobically at 37 ° C for 18 to 24 hours. Enterococcus isolates were selected by using Enterococcus selective media (Bile-esculin-azide agar (BEA) , Columbia blood agar and CHROMAagar) for 24 h at 35 °C in 5 % CO 2; salt tolerance test (6.5% NaCl),in order to differentiate

enterococci. The identification of Staphylococci or Enterococci isolates was based on standard laboratory criteria which the strains were identified by usual and biochemical tests allowing for discrimination among species: colony morphology, microscopic observation in the fresh state and after Gram staining, screening for catalase, production of staphylo-coagulase using rabbit plasma , and confirmation by using microbiological methods including API20 Staph or Strep system (Bio Merieux, France) .The automate identification of species was performed with the VITEK® 2 system (BioMerieux, Marcy l'Etoile, France), using Gram-positive identification (GP ID) cards.

Antibiotic susceptibility test:

Antibiotic susceptibility testing was performed by applying the method of discs diffusion in Mueller-Hinton agar using BioMerieux discs as recommended in guidelines established by the Antibiogram Committee of the French Microbiology Society (CA-SFM 2022) [7], and the results of some other discs were interpreted according to Clinical and Laboratory Standards Institute (CLSI) recommendations (2022) [8]. Confirmation of antimicrobial susceptibility was achieved by testing the minimum inhibitory concentration (MIC) and using the VITEK® 2 susceptibility system (BioMerieux, Inc., Durham, NC) AST-GP2 cards according to the manufacturer's instructions [9; 10].

The bacterial suspension was prepared by emulsifying bacterial isolates 1.5 x 10^7 CFU/ml in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard.

The disk diffusion method on Mueller–Hinton agar was performed with the following antibiotic disks: Penicillin G (PG-1U), Oxacillin (Ox-5µg), Cefoxitin (FOX-30 µg), Gentamicin (GM-10µg), , Amikacin (AK-30 µg), Tetracycline (TE-30µg), Erythromycin (E-15µg), Ofloxacin (OFX-5µg),Fusidic acid (FA-10 µg), Vancomycin (VA-30µg), Teicoplanin (TEC-30 µg), Clindamycin (CC-2 µg), Rifampicin (RA-5µg),and Trimethoprim-sulfamethoxazole (cotrimoxazole) (SXT-1.25 / 23.7 5µg). The minimum inhibitory concentrations (MICs) to vancomycin, teicoplanin, were determined by the E-test (bioMérieux, Marcy l'Etoile,France) method on Mueller-Hinton agar following the CLSI recommendations [8].

Microdilution susceptibility testing of isolates was performed using the Vitek ® 2 system (bioMérieux ® , Marcy L'Etoile, France). The following antibiotics were used: high-level gentamicin (HLG),erythromycin (ERY), clindamycin (CLI), high-level streptomycin (HLS),levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), quinupristin/dalfopristin (QD), tigecycline (TGC), vancomycin (VAN),teicoplanin (TEC), tetracycline (TET), trimethroprime+ sulfamethoxazol (SXT) ,and nitrofurantoin (NIT).

Phenotypic detection of methicillin resistance was applied by using method of oxacillin or Cefoxitin disk diffusion for detecting methicillin-resistant *Staphylococcus aureus* (MRSA) by An oxacillin disk (1 µg or 2 µg) on Mueller Hinton agar medium supplemented with 2% NaCl according to the directives of CLSI (2021) [8] ,followed by incubation at 35° C in ambient air and examined after 24 h. The growth of few colonies is sufficient to determine methicillin-resistance (12) or apply cefoxitin disk (30ug) (Liofilchem, Roseto degli Abruzzi, Italy) on MH agar. Whose diameter $\leq$ 21mm reads Methicillin resistance and diameter is $\geq$ 22mm indicates that is a Methicillin sensitivity according to the Clinical Laboratory Standard Institute (CLSI) guidelines [8].

The study of Antimicrobial susceptibility to glycopeptides (vancomycin, teicoplanin) was determined for each suspect strain (low sensitivity diameter) by evaluation of the Etest® quantitative minimum
inhibitory concentration (MICs).

The reference strains: *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used as quality control before every antibiogram (13)

Statistical Analyses

The data were analyzed using Statistical Package for Social Sciences (SPSS) software version 23.0 (International Business Machines Inc., Armonk, NY, USA). Demographic and clinical characteristics were summarized using absolute and percentage frequencies for the qualitative variables, and calculate the mean (or median) and standard deviation for the quantitative variables. Categorical variables were compared and identify the difference between the two ratios based on the Chi-square test. The t-test was used to compare the mean/median of the 2 groups (normally distributed),and the multi-group mean/median comparisons were based on the ANOVA test. Multivariate logistic regression analysis was used to identify risk factors associated with intestinal co-colonization with VRE and MRSA. This data analysis technique adopted to achieve the evaluation of the association between the Vancomycin resistance (qualitative explained variable) and the factors likely to influence it (the qualitative explanatory variables: sex, age, risk factor and medical wards). A value of $p < 0.05$ was determined to be statistically significant.

The links between the different variables were examined through the bivariate tables using significance tests such as Odds Ratio (OR), confidence intervals (CI) and P-value. The significance level was set at 95%. The association was said to be significant if the OR was greater than 1 and when the confidence interval did not contain 1. It was also significant if P was less than 0.05. The OR was used to determine the level of risk attributable to an F factor in the exposure of the population to an antibiotic-resistant germ. This association was qualified as significant when the confidence interval did not contain 1.

Results:

From August 2019 to August 2020, study cultures for 408 strains of Staphylococci and Enterococci were obtained from 24,642 hospitalized patients and outpatients.

In this survey, a total of 270 Staphylococcus isolates were identified from 405 strains obtained (66 %). One hundred twenty five of these isolates were *Staphylococcus aureus*. MRSA (*Methicillin resistant S.aureus)* strains constituted over 64% (n = 80) of all *S. aureus* isolates, 36% (n = 45) were MSSA (*Methicillin-sensitive Staphylococcus aureus)* ,and MRCNS(*Methicillin resistant coagulase negative staphylococci*) strains represented 53.32% of CNS strains. The highest prevalence of MRSA isolates was fou

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nd in ICU (P *=0.044) fellowed by* Internal medicine (26.3%), the female patients were more likely to have staphylococcal infections than the male patients in which the gender ratio (female/ male) was 1.2 (167/103). Deaths were recorded among 11(2.7%) patients. The staphylococcal isolates were obtained from 5 different specimens with the following percentage representations: pus (39.6 %), respiratory secretions (21.5%), blood (20%), tracheal (16.72%), urine (15.9%), vaginal/urethra swabs (5.2%).

In our study, the Antibacterial susceptibility testing results revealed a remarkable rate of antibiotic resistance .in this hospital, MRSA clearly appeared to be the predominant multidrug-resistant Staphylococci and exhibited pronounced resistance to the majority of antibiotics in which 46% of these strains were resistant to glycopeptides, more than 50% to rifampicin and nitrofurantoin, tetracycline, fusidic acid. Moreover, high resistance rates were observed for Ofloxacin (n = 67; 83.75%), pristinamycin (n = 72; 90%), and Lincomycin (n = 74; 92.5%). Inversely, gentamicin, cotrimoxazol were effective on the majority of MRSA strains. 5% out of these strains (4/80) were resistant to linezolid. (Table 2)

A total of 135 Enterococcus spp. strains (33.3%) were isolated during the study period. The two main clinical wards from where most enterococcal strains are isolated were the Internal medicine(47.4%)followed by the intensive care unit ICU(31.9%) from which 107 out of the 135 studied strains were collected. Of all enterococcal isolates, Enterococcus faecalis were the most predominant isolates representing 60% (n=81/135), followed by E. faecium (n=52/135, 38.5%), and the less commonly founded, Enterococcus sp (n=2/135,1.5%).The bacterial isolates were obtained from 5 different specimens with the following proportion representations: urine (45.9%),blood (35.6%), pus (8.1%), vaginal/urethra swabs (3%), respiratory secretions (2.2%). E. faecalis (n = 61; 75. 3%) was the predominant species detected in female sex with the average age of patients 50 years (51 to 89years), while the majority of patients whose E. faecium (n = 39) was isolated were male with the average age of patients 53.1 years (51 to 89years), Among enterococcal strains, vancomycin-resistant Enterococci were observed in 25.9% (35/135). Out of these 35 isolates, 23 (17%) were Enterococcus faecium and 12 (8.9%) were Enterococcus faecalis. 23 out of 52 isolates of E. faecium (44.2%) and 13out of 81 E.faecalis (16%) were found to be co-colonized with Staphylococci. However, two strains of vancomycin-resistant E.faecalis and E. faecium were detected in the same patient

All the 35 vancomycin-resistant Enterococcal strains showed high level of resistance to vancomycin

(MICs 32-256 µg/ml) and teicoplanin (MICs 16- 256µg/ml). in addition to glycopeptides, all these strains were MDR (resistant to more than three classes of antimicrobial agents) and presented levels of resistance rates higher than 85% to erythromycin, quinupristin-dalfopristin, tetracycline, levofloxacin, high-level gentamicin and high-level streptomycin, Cotimoxazol ,Nitrofurantoin . However, tigecycline, Linezolide showed good activity against VRE strains with MICs $\leq$ 3 µg/ml and $\leq$ 8 µg/ml respectively. (table2)

Prevalence of co-existence with MRSA and VRE

Our concept in this section of research is the retrospective study of 2 groups (case-control): one with patients who have a co-infection by staphylococci precisely those of MRSA and enterococci in particular VRE by acting as cases and the other group of patients with only Staphylococcus or enterococcus only acting as controls. In this study, According to the principle of this distribution, a total of 368 patients were identified as having Staphylococcus or enterococcus infection, while, we isolated both MRSA and VRE from thirty seven Patients during the hospital stay. Demographic and clinical data of patients are shown in Table 1. The main age of patients harboring both MRSA and VRE was 57years (IQR 51-89years)(figue1).

The rate of nosocomial co- infections due to VRE with MRSA is much higher in intensive care units (p-value=*0.044538* <0.05 / Odds ratio of 17.45) with a significant mortality compared to other

strains .Pus +6++specimens were the most common among the 37 single specimens that yielded both organisms (45.9%), followed by blood specimens (40.5%), and urine specimens (13.5%). There was a statistically significant difference between these groups related to co-morbidities such as cancer, diabetes, Chronic renal disease, respiratory disease (Covid 19) .About 24.3% of co-infected patients died of which odds ratio of mortality was OR, 2.071 (95% CI, 1.022-2.225)

Table 1. Demographic and clinical data of patients

Charasteristics	***Enterococcus* or *Staphylococcus* (%)**	***Enterococcus* + *Staphylococcus* (%)**	**P-value**
Total of patients	*368*	*37*	
demographic data			
Male/Female, sex ratio	146/222	18/19	0,824
Age (y), mean	57 (51-89)	67(51-89)	0,99
Clinical outcome			
Mortality rate	4(1,6)	9(24,3)	<0,001
Isolation site			
blood	103(26,1)	24(5,9)	0,025
pus	110(27,2)	8(2)	0,492
urinary tract	105(25,9)	2(0,5)	0,069
respiratory secretions	50(12,3)	3(0,7)	0,049
Clinical wards			
Internal medicine	106(26,1)	5(1,2)	0,009
Intensive care unit	66(16,3)	26(6,4)	<0,001
pediatric	66(16,3)	2(0,5)	0,69
Cardiology	51(12,6)	2(0,5)	0,045
Surgery	50(12,3)	1(0,2)	0,66
outpatient	29(7,2)	0	0,789
Comorbidities			
cancer	59(16,03)	6(1,62)	0,025
Covid19	75(20)	20(54)	<0,001
Diabetes mellitus	179(48,64)	20(54)	<0,001
renal failure	69(18,75	13(35,1)	<0,001
cardiac	118(32)	11(29,7)	0,039

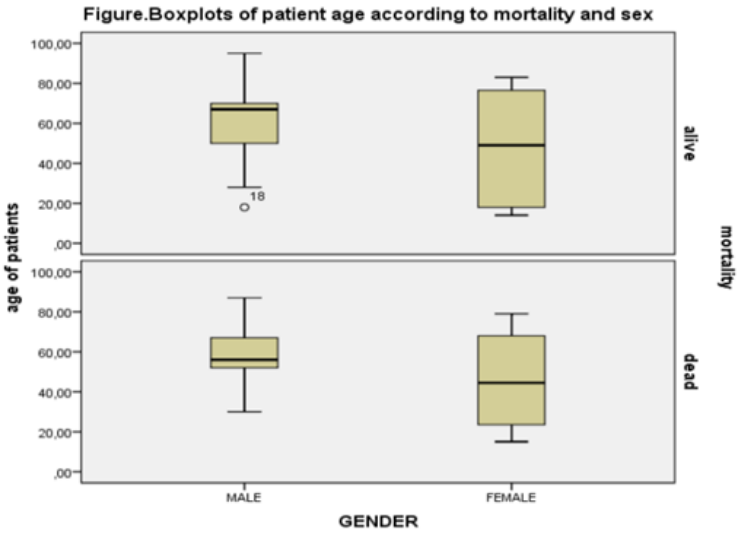
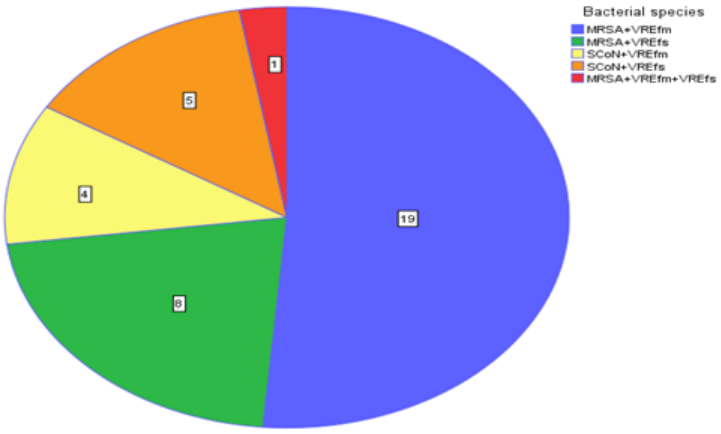
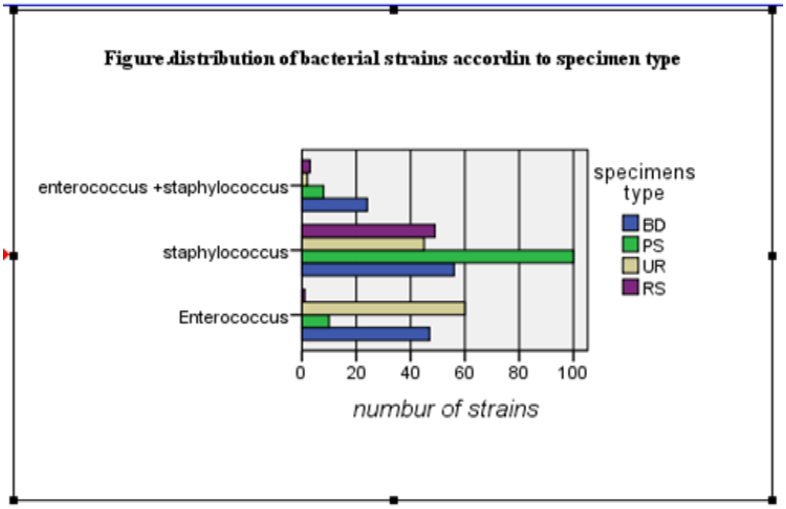


Figure. distribution of strains according to bacterial species





Bacterial species	specimens type							
	BD		PS		UR		RS	
	N	Nb. (%)	N	Nb. (%)	N	Nb. (%)	N	Nb. (%)
E feacalis	23	5,7%	8	2,0%	41	10,1%	0	0,0%
E faecium	24	5,9%	2	0,5%	19	4,7%	1	0,2%
MRSA	12	3,0%	49	12,1%	1	0,2%	3	0,7%
MSSA	17	4,2%	12	3,0%	10	2,5%	2	0,5%
S CoN	27	6,7%	39	9,6%	34	8,4%	44	10,9%
MRSA+VREfm	11	2,7%	7	1,7%	0	0,0%	1	0,2%
MRSA+VREfs	7	1,7%	0	0,0%	1	0,2%	0	0,0%
SCoN+VREfm	3	0,7%	0	0,0%	0	0,0%	1	0,2%
SCoN+VREfs	2	0,5%	1	0,2%	1	0,2%	1	0,2%
MRSA+VREfm+VREfs	1	0,2%	0	0,0%	0	0,0%	0	0,0%

Table. Antimicrobial resistance patterns of bacterial isolates

Antimicrobial agents tested		**bacterial isolates**					
		Enterococcus		*Staphylococcus*		*Enterococcus +Staphylococcus*	
		N	Nb (%)	N	Nb. (%)	N	Nb. (%)
Benzylpenicillin	R	46	28,4%	86	53,1%	28	17,3%

Oxacillin	R	46	28,4%	86	53,1%	28	17,3%
Gentamycin	R	70	57,4%	28	23,0%	22	18,0%
Kanamycin	R	118	58,7%	50	24,9%	31	15,4%
Tobramycin	R	118	63,4%	39	21,0%	27	14,5%
Ofloxacin	R	0	0,0%	52	83,9%	8	12,9%
Clindamycin	R	118	47,8%	93	37,7%	34	13,8%
Erythromycin	R	118	34,6%	187	54,8%	34	10,0%
Lincomycin	R	118	48,0%	93	37,8%	33	13,4%
Pristinamycin	R	118	47,8%	93	37,7%	34	13,8%
Linezolid	R	0	0,0%	21	77,8%	4	14,8%
Teicoplanin	R	0	0,0%	10	40,0%	19	52,0%
Vancomycin	R	0	0,0%	7	25,0%	37	67,9%
Tetracyclin	R	109	32,2%	191	56,3%	37	10,9%
Fosfomycin	R	0	0,0%	47	77,0%	12	19,7%
Nitrofurantoin	R	40	32,0%	61	48,8%	22	17,6%
fusidic acid	R	0	0,0%	81	81,0%	17	17,0%
Rifampecin	R	92	38,5%	112	46,9%	33	13,8%
Cotrimoxazol	R	103	56,0%	50	27,2%	29	15,8%
Ampecillin	R	46	43,8%	39	37,1%	18	17,1%
Quinipristindalfopristin	R	105	86,8%	0	0,0%	14	11,6%
Levoffloxacin	R	59	81,9%	0	0,0%	11	15,3%
R: resistant ;N number of strains							

Discussion

The co colonization or co infection by MRSA and VRE which leads to the therapeutic impasse caused by the transfer of resistance is the main threat to public health and the fight against the appearance of highly resistant strains is one of the challenges of our recent life. The foremost risk factors for the proximity of VRE to MRSA within a single specimen is may facilitate the transfer of the vanA gene from VRE to MRSA. The highest occurrence of both strains in a single specimen was detected in the ICU with P-value <0.001.

The 250 isolates of Staphylococci were isolated mainly from pus (n=100; 40%), followed by blood (n=56; 22.4%), 19.6%, and 18% of these isolates came from respiratory secretions and l urine respectively. Our strains are found mainly in pus samples of all origins, followed by blood cultures. This result agrees well with that study of Flannery et *al*. Carried out in 2011 **(13).** It is also close to that of Aouati's study which reports an isolation percentage from pus samples of 62% and 30% for blood cultures **(14).** These bacteria can colonize the skin asymptomatically. When skin lesions, wounds, or entry points develop, these bacteria have the opportunity to enter tissues and cause infection **(16).** As it can also be explained by the secretion of toxins of which, some staphylococci, particularly *S. aureus*, produce toxins that can damage tissues, cause inflammation and contribute to the characteristic symptoms of staphylococcal infections, such as abscesses and pus **(15).** Our enterococci strains are found mainly in urine samples (50.8%) and blood (39.8%), followed by purulent samples with a rate of less than 9%. This distribution is higher than that observed in a recent study carried out in hospitals in southeastern Romania by Iancu et al. by isolating 38.4% and 32.6% of bacterial strains in urine and blood cultures respectively **(18)**. Our results were comparable to those found by El Ghazawy et al who reported rates of 64.6% of isolates came from urine samples (**19**).

While Yadav and Agarwal found a percentage highest (86.2%) of samples of urinary origin **(20).** This is consistent with enterococci being one of the main causes of urinary tract infections due to the increased and prolonged use of urinary catheters in hospitals. On the other hand, enterococci are capable of adapting to a wide range of environmental conditions, including those present in the urinary tract. Their ability to survive in a relatively hostile environment may contribute to their predominance in the urine when an infection develops and in this case, this resistance may limit the effectiveness of traditional antibiotic treatments **(21)**. While the thirty-seven strains resulting from co-infection with Staphylococci plus Enterococci are mainly obtained during a COVID-19 pandemic, where seriously affected patients may have a weakened immune system, making them more vulnerable to bacterial infections leading to sepsis (bacteremia or serious infections) and in this case the infection can spread quickly through the blood system, allowing different strains of bacteria to colonize the blood. Additionally, patients with severe COVID-19 may have a weakened skin barrier due to medical interventions such as intubations, catheters, etc. This may facilitate the entry of pathogenic bacteria, including MRSA and ERG, into the bloodstream, which could be detected by an elevated level in blood cultures **(17).**

In our study, independent risk factors for co-colonization or coinfection with VRE and MRSA were age due to changes in the immune system, male gender, prior hospitalization, residing in a long-term care facility, Prolonged ICU stays especially increase the exposure to hospital-acquired infections., exposure to invasive medical devices, renal insufficiency, excessive use of antimicrobial agent which leads to increase in the rate of multidrug resistance

The analysis of resistance profile of our strains founded in our study confirms the multiresistant nature of these bacteria to different families of antibiotics which were highly resistant against to one or more classes of antibiotics. The study of antibiotic susceptibility of of MRSA determined that the rate of resistance to oxacillin, cefoxitin and penicillin G is of 100%, this rate is the same as that found by Rebiahi and *al* **(22);** Touaitia and *al*(**23)**

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