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Abstract

The spread of vancomycin-resistant Enterococci (VRE) in Algerian hospital settings is poorly reported. Since the first report in 2006, few data have been available on the molecular mechanism of this resistance across the country. In this study, we investigate the frequency and antibiotic resistance mechanisms of *Enterococci* strains isolated from hospitalised patients in the Tlemcen university hospital. 191 *Enterococcus* spp. strains were collected from various clinical samples and were identified using MALDI-TOF-MS. The presence of *van* genes was investigated by standard PCR and sequencing. Results revealed that *E. faecium* and *E. faecalis* strains are the main pathogens identified in the study. Antibiotic susceptibility testing revealed that the resistance rate was high for the majority of antibiotic classes, including glycopeptides, and only linezolid was effective on all strains. Molecular analysis revealed that 52.2% of strains from intensive care unit (ICU) were positive for the *vanA* gene, including 44.44% *E. faecium*, 5.55% *E. faecalis* and 2.22% *E. avium.* 25.5% of these isolates co-harboured both the *vanA* and *vanC* genes, including *E. gallinarum* (n=16) and *E. faecium* (n=6). In surgical wards (SW) 29.70% of strains harboured the *van* genes, including 4.90% of *E. faecalis* harbouring the *vanB* gene, and of the rest of strains, (24.80%) harboured the *vanC* genes. Indeed, 9.90% *E. gallinarum* and 4.90% *E. faecalis* were positive for *vanC1* and 9.90% of *E. casseliflavus* were positive for the *vanC2/C3* gene. The glycopeptide resistance rate was higher among strains from the ICU and was mainly composed by *E. faecium* strains compared with surgical wards where resistant *E. faecalis* strains were predominant.

Introduction

Mostly *E. faecium*, and to some extent *E. faecalis*, are involved in healthcare-associated infections and nosocomial outbreaks. These can cause serious infections ranging from urinary tract infections to endocarditis, meningitis or bacteraemia, which are sources of significant morbidity and mortality (O'Driscoll and Crank, 2015). Enterococci are naturally resistant to many routinely used antibiotics such as cephalosporins, low concentrations of aminoglycosides, clindamycin, fluoroquinolones, trimethoprim-sulfamethoxazole (Cetinkaya *et al.*, 2000). They are characterised by particular genome plasticity allowing them to resist other antibiotics (Bender *et al.*, 2018). Vancomycin, considered as drug of last resort, has long been used against Gram-positive bacteria which are resistant to β-lactams responsible for serious infections including sepsis, meningitis, endocarditis, pneumonia, enterocolitis and osteomyelitis (Rivera and Boucher, 2011). Vancomycin-resistant Enterococci (VREs) are easily transmitted through asymptomatic patients, caregivers, and contaminated environments or instruments (Escaut *et al.*, 2013). VREs can persist on inanimate surfaces for a long time and show a remarkable ability to colonise gastrointestinal (GI) tracts of hospitalised patients (Vehreschild *et al.*, 2019). An imbalance in hospital hygiene coupled with the excessive use of broad-spectrum antibiotics frequently results in VRE nosocomial outbreaks in high-risk departments such as transplant departments (Kreidl *et al.*, 2018), oncology (Oh *et al.*, 2004) and intensive care units (ICU) (Hughes *et al.*, 2019). As reported, the resistance mechanism to vancomycin is mediated by the modification of vancomycin-binding targets leading to alterations in the peptidoglycan synthesis. Indeed, the terminal d-Ala-d-Ala peptide of N-acetylmuramic acid is substituted with d-Lac or d-Ser, causing a significant decrease in the affinity between cell wall precursors and the antibiotic (Hollenbeck and Rice, 2012). Eight gene variants (*vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*) have been reported as acquired genes conferring resistance to vancomycin and only the *vanC* gene appears to be an

intrinsic gene of *E. gallinarum* and *E. casseliflavus*, and *E. flavescens* (Faron *et al.*, 2016). The dominant VRE clone harbouring *vanA* has been responsible for numerous hospital outbreaks worldwide (Rangberg *et al.*, 2019) (Douglas *et al.*, 2019) but, an emerging clone harbouring *vanB* has also been reported (Hughes *et al.*, 2019) (Coombs *et al.*, 2014).The management of VREs infections poses a real challenge for clinicians, as the therapeutic arsenal is often limited to linezolid (Dotis *et al.*, 2010). Active surveillance based on screening and isolation of colonised or infected patients with rigorous disinfection remains the cornerstone of measures to control and limit transmission and the occurrence of outbreaks (Kreidl *et al.*, 2018). In Algerian hospitals, only a few studies have reported VRE strains, harbouring only the *vanA* gene (Benammar *et al.*, 2018; Djahmi *et al.*, 2012).

In this study, we investigate the rate and molecular mechanisms of glycopeptide and linezolid resistance among clinical *Enterococcus* strains isolated from hospitalised patients at the Tlemcen university hospital in Algeria.

Materials and methods

Bacterial strains:

Between October 2016 and June 2017, a total of 191 clinical *Enterococcus* strains were isolated from various specimens (bronchial aspirates, gastric aspirates, ascitic fluids, cerebrospinal fluids, vaginal discharges, urine and wounds) collected from hospitalised patients in the intensive care unit (ICU) and surgical wards (SW), including Urology, Emergency Surgery, Orthopaedic-Trauma Surgery, General Surgery, and Neurosurgery at the Tlemcen university hospital, comprising a total of 646 beds.

Bacterial identification

Specimens were plated onto colistin nalidixic acid (CNA) agar with 5% Sheep Blood (bioMérieux, Marcy-l'Étoile, France) and incubated at 37°C for 48 hours. All *Enterococcus*

spp. strains were identified using MALDI-TOF/MS (MicroflexTM; Bruker Daltonik GmbH, Bremen, Germany) as previously described (Seng *et al.*, 2009).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the disk diffusion method according to the recommendations of CA-SFM (Antibiogram Committee of the French Microbiology Society, 2019). Sixteen antibiotics were tested, including penicillin G, oxacillin, amoxicillin, ceftriaxone, clindamycin, erythromycin, pristinamycin, gentamicin, vancomycin, teicoplanin, doxycycline, minocycline, nitrofurantoin, linezolid, rifampicin, fosfomycin, (all from i2a, Montpellier, France). Minimum inhibitory concentrations (MICs) of vancomycin, teicoplanin, and linezolid were determined by the E-test method (bioMérieux, Marcy-l'Étoile, France) and interpreted in accordance with CA-SFM guidelines.

Molecular analysis

Standard PCR and sequencing was performed to confirm and screen for the presence of *vanA*, *vanB*, *vanC1*, *vanC2/C3* genes, as previously described (Domingo *et al.*, 2005) (Said and Abdelmegeed, 2019). All the primers used are presented in **(Suppl. Table S1).**

Results

Clinical Data: The average age of patients in the ICU was 54.1 years (22 to 82 years), and 51.3 years (20 to 86 years) in the SW. All patients had received antibiotic treatment for at least five days and had indwelling devices. In the ICU, all patients were receiving mechanical ventilation and the average length of their hospital stay was more 15 days [7-150], compared to only seven days [5-18] in SW patients. Demographic and clinical data of patients are shown in **Table 1**.

Bacterial strain identification: A total of 191 *Enterococcus* spp. strains were isolated during the study period. 90 out of the 191 strains were recovered from ICU samples and 101 were obtained from SW samples. Bronchial aspirates and wounds were the most common samples from the ICU (35.55%) and SW (41.58%) strains, followed by urine samples from both departments **(Figure 1)**.

As shown in **Figure 1A,** the ICU strains consisted of five different species of which *E. faecium* (n= 48; 53.33%) was the most frequently isolated followed by *E. faecalis* (n=18)*, E. gallinarum* (n= 19)*, E. avium* (n=4)*, E. casseflavus* (n=1). In contrast, the SW strains consisted of six different species and *E. faecalis* (n=47; 46.53%) was the predominant species, while the rest was composed of *E. faecium* (n=25), *E. casseliflavus* (n=11), *E. gallinarum* (n=9), *E. hirae* (n=8), and *E. otavius* (n=1) **(Figure 1B)**. Interestingly, bronchial aspirates and surgical wounds from both departments were the two clinical specimens from which 38.74% of the studied strains (74/191) were collected.

Antibacterial susceptibility testing (AST): Surprisingly, the AST results revealed an interesting distribution of antibiotic resistance in this hospital. Indeed, as shown in **Figure 2**, in the ICU, with the exception of linezolid, *E. faecium* clearly appeared to be the predominant multidrug-resistant Enterococci in this ward. *E. faecium* exhibited pronounced resistance to

the majority of antibiotics such as β-lactams, vancomycin, teicoplanin, clindamycin, erythromycin, gentamicin, and fosfomycin. 96% of these *E. faecium* strains were resistant to glycopeptides, 56% and 46% to rifampicin and nitrofurantoin respectively and, inversely, doxycycline, minocycline, and pristinamycin were effective on the majority of *E. faecium* strains. None of the *E. faecium* strains were resistant to linezolid (**Fig. 2A**). Moreover, five *E. faecalis* strains were found to be resistant to glycopeptides (vancomycin and teicoplanin) and 15 out of the 18 *E. faecalis* from this ICU were resistance to oxacillin, ceftriaxone, clindamycin, erythromycin, gentamycin, and fosfomycin (**Fig. 2A**). Other *Enterococcus* species such as *E. gallinarum* also exhibited a multidrug resistance phenotype in this ICU.

In contrast to the ICU, in the surgical wards, *E. faecalis* was the predominant MDR pathogen identified. Indeed, 45 out of the 47 *E. faecalis* (95.74%) strains were fully resistant to penicillin G, oxacillin, ceftriaxone, and clindamycin (**Fig. 2B**). Moreover, high resistance rates were observed for erythromycin (n=23; 48.93%), pristinamycin (n=33; 70.21%), and fosfomycin (n=26; 55.32%). The resistance rate to doxycycline was 34% (n=16), while none were resistant to minocycline. Interestingly, none of the identified *E. faecium* strains in the SWs were resistant to glycopeptides (vancomycin and teicoplanin) (**Fig. 2B**). However, all of the 25 (100%) *E. faecium* strains were resistant to β-lactams (i.e. penicillin G, oxacillin, amoxicillin, and ceftriaxone), 17 (28%) were resistant to clindamycin, 14 (56%) were resistant to fosfomycin, and 10 (40%) were resistant to rifampicin (**Fig. 2B**). As observed for strains from the ICU, none of the strains from the SWs were resistant to linezolid (MIC < 2 µg/mL) (**Fig. 2 A and B**).

Molecular analysis: Very surprisingly, the resistance level to glycopeptides (vancomycin and teicoplanin) was significantly different when comparing both departments. It is well established that the *vanA* gene confers a high MIC to vancomycin and teicoplanin and that the *vanB* gene confers resistance only to vancomycin. Indeed, in strains from the

ICU, a very high level of glycopeptide resistance (i.e., MIC_{VAN} > 256 µg/mL and MIC_{TEC} > 256 µg/mL) was observed for all resistant strains (**Table 2**); while this resistance level to glycopeptides in strains from the SWs ranged from 4 to 32 µg/mL, with the exception of five strains with MIC_{VAN} = 128 µg/mL (**Table 3**). Interestingly, these findings align perfectly with the molecular investigation of glycopeptide resistance genes, including *vanA, vanB, vanC* gene variants in all resistant *Enterococci* strains. In fact, molecular analysis targeting the *van* gene variants has revealed that the high resistance level observed in the ICU strains was clearly associated with the presence of the *vanA* gene in 70 out of the 90 strains in different species (**Table 2**). Its distribution in the ICU strains was as follows: *E. faecium* (n = 46), *E. faecalis* (n = 5), *E. gallinarum* (n= 16), *E. avium* (n=2) and *E. casseliflavus* (n =1). The simultaneous presence of *vanA*/*vanC* was identified in 24.4% (n=22 strains). In the SWs, *vanB* and *vanC* gene variants were mainly identified in strains and were associated with the observed resistance level in this ward (**Table 3**). In this ward, the *vanC*1 gene was detected in five *E. faecalis* and ten *E. gallinarum* strains and the simultaneous presence of *vanC*2/*vanC*3 was identified in ten *E. casseliflavus* strains. No *vanA* genes were detected in the SW strains (**Table 3**).

Discussion

Since their first detection in 1978, *E. faecium* vancomycin-resistant, and to some extent *E. faecalis* vancomycin-resistant strains have spread worldwide and are responsible for nosocomial outbreaks, substantial mortality, and additional costs to healthcare facilities every year (Deshpande *et al.*, 2007; Simner *et al.*, 2015) . In 2017, the World Health Organization (WHO) classified vancomycin-resistant *E. faecium*- in a list of high priorities, requiring research and the development of new effective antibiotics (Markwart *et al.*, 2019). In Algeria, the Algerian Antimicrobial Resistance Network (ARN) reported a low rate of vancomycin-resistant *E. faecium*- and vancomycin-resistant *E. faecalis*-

(http://www.sante.dz/aarn/rapports.htm). Between 2017 and 2018, ARN reported low VRE percentages in Algeria, with 1.9% for *E. faecalis* and 24.4% for *E. faecium*. These national surveillance data are not exhaustive, however, since they do not report the genetic support of the glycopeptide resistance mechanism and concern only ten Algerian hospitals. In 2006, the first VRE strain identified was an *E. faecalis* strain from a urine sample taken in the Beni-Messous university hospital in Algiers (Aggoune *et al.*, 2008). However, a limited number of molecular studies in Algeria to date have reported the resistance mechanism to glycopeptides. One such study described five strains of *vanA*-producing *E. faecium* from the centre and east of country, more specifically from the university hospital in Algiers (Hamidi *et al.*, 2019) and the Batna university hospital (Benammar *et al.*, 2018).

Here we report, for the first time, a high rate (52.35%) of clinical VRE strains in western Algeria, including strains carrying the *vanB* gene. The vast majority of VREs (77.7%) were found in the ICU and only a few strains were revealed in samples taken from SWs. The ICU admits critically-ill patients for extended stays (Cetinkaya *et al.*, 2000), and as such is one of the largest wards, and largest consumers of broad-spectrum antibiotics such as glycopeptides and third-generation cephalosporins in our hospital, which explains the high rate of resistance strains associated with the presence of *vanA* resistance gene. Linezolid appears to be the only effective drug against all *Enterococcus* spp. isolated in this study and could be an exciting therapeutic option. A high rate of *E. faecium* was identified in the ICU compared to the SWs, where *E. faecalis* was the most predominant. Their distribution differs according to departments, specimen types, and geographical locations (Kolář, 2018). Pronounced resistance to glycopeptides is often detected in *E. faecium*, elucidating the high rate of VRE strains in our ICU mediated by the *vanA* gene.

A considerable number of *E. gallinarum* and *E. casseliflavus* strains were identified in this study, which have been sporadically found in clinical specimens and were often

misidentified or underestimated in the past (Monticelli *et al.*, 2018). Despite their low pathogenicity, these strains are increasingly incriminated in invasive infections in immunocompromised patients (Choi *et al.*, 2004; Reid *et al.*, 2001), and even nosocomial outbreaks (Contreras *et al.*, 2008). As reported, *E. gallinarum* and *E. casseliflavus* can acquire transferable plasmids carrying vancomycin-resistance genes such as the *vanA* or *vanB* gene (Haenni *et al.*, 2009; Kateete *et al.*, 2019). In this study, 16 *E. gallinarum* and one *E. casseliflavus* strains harboured the *vanA* gene. The presence of VREs must be rapidly controlled to avoid spreading resistant strains among patients and to prevent hospital outbreaks (Cetinkaya *et al.*, 2000). Our study reports the first *E. faecalis* strains carrying the *vanC1* gene originating from human clinical specimens. The *vanC* genotype has only been revealed in small number of *E. faecalis* strains isolated from animal samples, including pig manure (Germany)(Schwaiger *et al.*, 2012), sheep milk (Spain)(de Garnica *et al.*, 2013) and cloacal swabs from broilers (Brazil) (Moura *et al.*, 2013). The detection of *vanC1* genes in *E. faecalis* and *E. faecium* is remarkable because this gene is intrinsic to *E. gallinarum* and has been used for species identification (Schwaiger *et al.*, 2012). Previous reports have demonstrated that *E. faecium* and *E. faecalis* strains can acquired the *vanC* gene by horizontal transfer from *E. gallinarum* in animal guts (Moura *et al.*, 2013) and in aquatic environments (Nishiyama *et al.*, 2015). These observations underline a considerable ability that Enterococci strains have to exchange resistance genes.

As reported in the literature, *vanA* and *vanB* genes are most common encountered in human clinical specimens and global nosocomial outbreaks and, thus, must be monitored as suggested (Freitas *et al.*, 2016; Hughes *et al.*, 2019).

In conclusion, our findings highlight a high incidence of VREs in the intensive care unit at the Tlemcen university hospital. A range of resistance mechanisms is currently emerging in our hospitals. These changes are due to epidemiological evolution in the country.

Permanent surveillance must be ensured to avoid the spread of resistance strains. Massive testing of high-risk patients, isolation of positive cases, and appropriate disinfection have always proven to be effective in preventing and managing outbreaks.

Ethical issues

All rules of confidentiality and ethics as prescribed in the Helsinki Declaration have been respected. Prior to data collection, the respective authorisations from the Tlemcen University Hospital's medical director and the prevention and control infections team were obtained. Data were collected anonymously.

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Figure legends

Figure 1. Distribution of clinical *Enterococcus* species isolated from different types of infection. **1A:** number of isolated species from the ICU. **1B:** number of isolated species from SWs.

Figure 2: Comparison of antimicrobial resistance of clinical *Enterococcus* strains from the ICU and SWs. *Enterococcus* spp. regrouped other *Enterococcus* species including *E. avium* (n=4), *E. casseflavus* (n=1), *E. gallinarum* (n= 19) from the ICU and *E. casseliflavus* (n=11), *E. octavius* (n=1), *E. gallinarum* (n=9), *E. hirae* (n=8) from SWs.

Table 1: Summary of demographic and clinical data of the study patients

	Demographic and clinical data	ICU patients n(%) N= 75	Surgical patients n(%) N= 101
Demographics	Age (years), [range]	54,14 [22-82]	51,28 [20-86]
	Male gender	43 (57,33%)	56 (55%)
Underlying diseases	Cardiovascular disease	38 (51%)	56 (55%)
	Respiratory disease	21 (28%)	7 (7%)
	Hepatobiliary disease	4 (5%)	27 (27%)
	Genitourinary disease	9 (12%)	8 (8%)
	Gastroenterological disease	3 (4%)	27 (27%)
	Autoimmune disease	8 (11%)	5 (5%)
	Malignancy	23 (31%)	29 ((29%)
	Chemotherapy	10 (13%)	32 (32%)
	Recent surgery	27 (36%)	101 (100%)
	Endocrinologic comorbidity	43 (57%)	80 (79%)
	Dialyze	25 (33%)	12 (12%)
Antibiotics administered	Carbapenems	57 (76%)	32 (32%)
	Quinolones	38 (51%)	64 (63%)
	Cephalosporins	75 (100%)	56 (55%)
	β-lactam/lactamase inhibitors	23 (31%)	57 (56%)
	Aminoglycosides	53 (71%)	78 (77%)
	Glycopeptides	52 (69%)	24 (24%)
	Metronidazole	16 (21%)	37 (37%)
	De-escalation	75 (100%)	43 (43%)
Indwelling device	Central venous catheter	75 (100%)	25 (25%)
	Urinary catheter	75 (100%)	89 (88%)
	Arterial catheter	75 (100%)	101 (100%)
	Mechanical ventilation	75 (100%)	0 (0%)
Hospitalisation data	Hospital stay, days, median [range]	15 [7-150]	7 [5-18]
	Prior hospitalisation	58 (77%)	28 (28%)
	Transferred from other hospitals,	17 (23%)	23 (23%)
	Transferred from other service	32 (43%)	33 (33%)
	Polytrauma	37 (49%)	5 (5%)
	Emergency surgery	28 (37%)	56 (55%)

Table 2: The distribution of vancomycin, teicoplanin MIC range, and resistance genes of isolated species from the ICU

Species name	Number of strains	No. of VanR (MIC)	No. of TecR (MIC)	*vanA*	*vanA-vanC*1	*vanA-vanC*2/*C*3
E. avium	4	2 (> 256 µg/mL)	2 (> 256 µg/mL)	2	0	0
E. casseflavus	1	1 (> 256 µg/mL)	1 (> 256 µg/mL)	0	0	1
E. faecalis	18	5 (> 256 µg/mL)	5 (> 256 µg/mL)	5	0	0
E. faecium	48	46 (>256 µg/mL)	46 (>256 µg/mL)	40	6	0
E. gallinarum	19	16 (>256 µg/mL)	16 (>256 µg/mL)	0	16	0
Total	90	70	70	47	22	1

VanR. vancomycin- resistance, TecR. Teicoplanin resistance, No. number of strains, MIC minimum inhibitory concentration. The *vanA*, *vanA-vanC*1 and, *vanA-vanC*1-*vanC*2/*C*3 genes were detected only in *Enterococci* spp. isolated from the ICU and the *vanB*, *vanC*1 and, *vanC*2/*C*3 genes were detected only in Enterococci spp. isolated from SWs.

Table 3: The distribution of vancomycin, teicoplanin MIC range, and resistance genes of isolated species from SWs.

Species name	No of strains	No. of VanR (MIC)	No. of TecR (MIC)	*vanB*	*vanC*1	*vanC*2/*C*3
E. casseliflavus	11	10 (4-28 µg/mL)	10 (4-28 µg/mL)	0	0	10
E. otavius	1	0	0	0	0	0
E. faecalis	47	10 (4-128 µg/mL)	10 (4-32 µg/mL)	5	5	0
E. faecium	24	0	0	0	0	0
E. gallinarum	10	10 (4-32 µg/mL)	10 (4-32 µg/mL)	0	10	0
E. hirae	8	0	0	0	0	0
Total	**101**	**30**	**30**	**5**	**15**	**10**

VanR. vancomycin- resistance, TecR. Teicoplanin resistance, No. number of strains, MIC minimum inhibitory concentration. The *vanA*, *vanA*-*vanC*1 and, *vanA*-*vanC*1-*vanC*2/*C*3 genes were detected only in *Enterococci* spp. isolated from the ICU and the *vanB*, *vanC*1 and, *vanC*2/*C*3 genes were detected only in *Enterococci* spp. isolated from SWs.

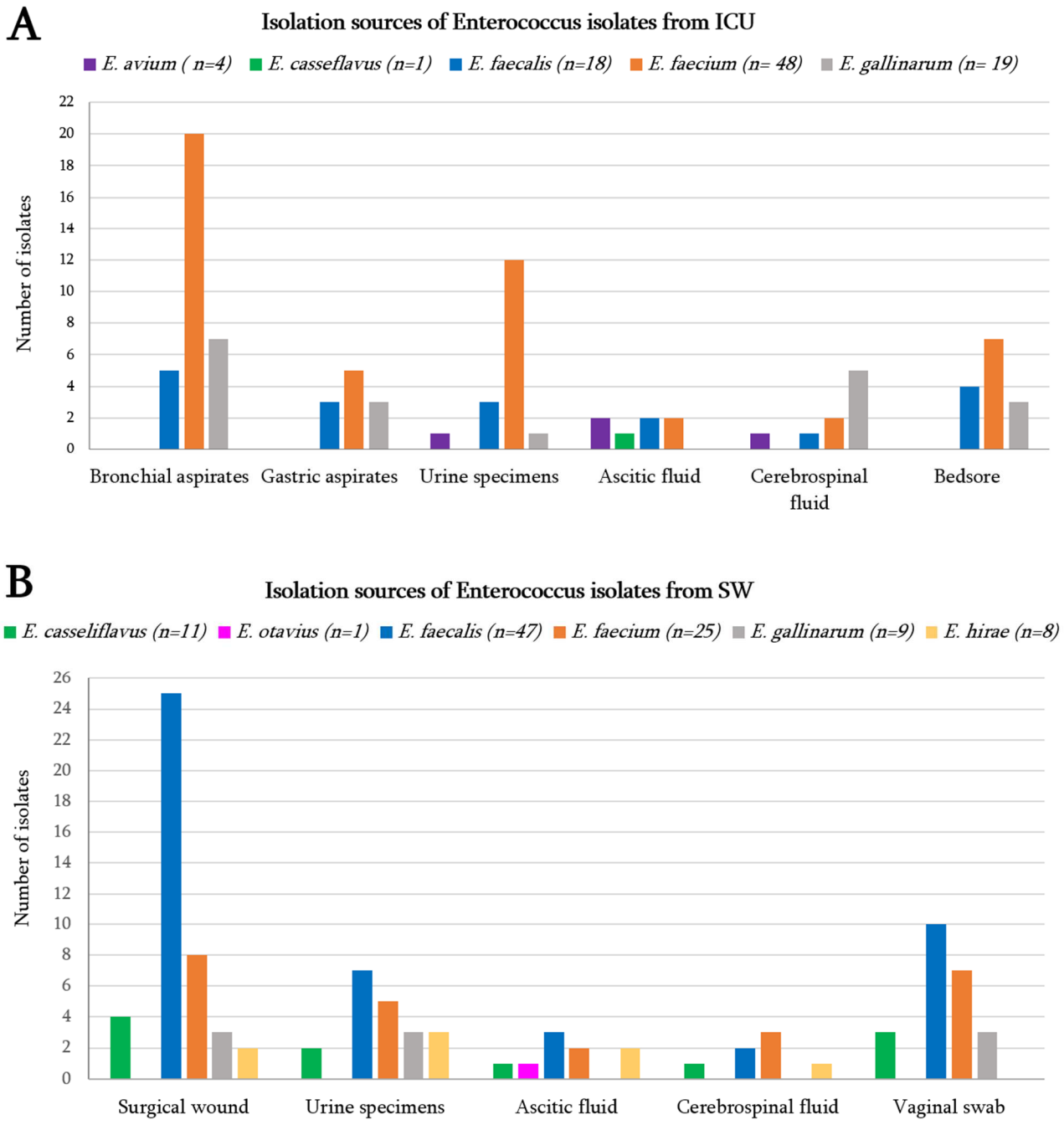
A
Isolation sources of Enterococcus isolates from ICU
E. avium (n=4)
E. casseflavus (n=1)
E. faecalis (n=18)
E. faecium (n= 48)
E. gallinarum (n= 19)
Number of isolates
0
2
4
6
8
10
12
14
16
18
20
22
Bronchial aspirates
Gastric aspirates
Urine specimens
Ascitic fluid
Cerebrospinal fluid
Bedsore
B
Isolation sources of Enterococcus isolates from SW
E. casseliflavus (n=11)
E. otavius (n=1)
E. faecalis (n=47)
E. faecium (n=25)
E. gallinarum (n=9)
E. hirae (n=8)
Number of isolates
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Surgical wound
Urine specimens
Ascitic fluid
Cerebrospinal fluid
Vaginal swab

Figure 1.

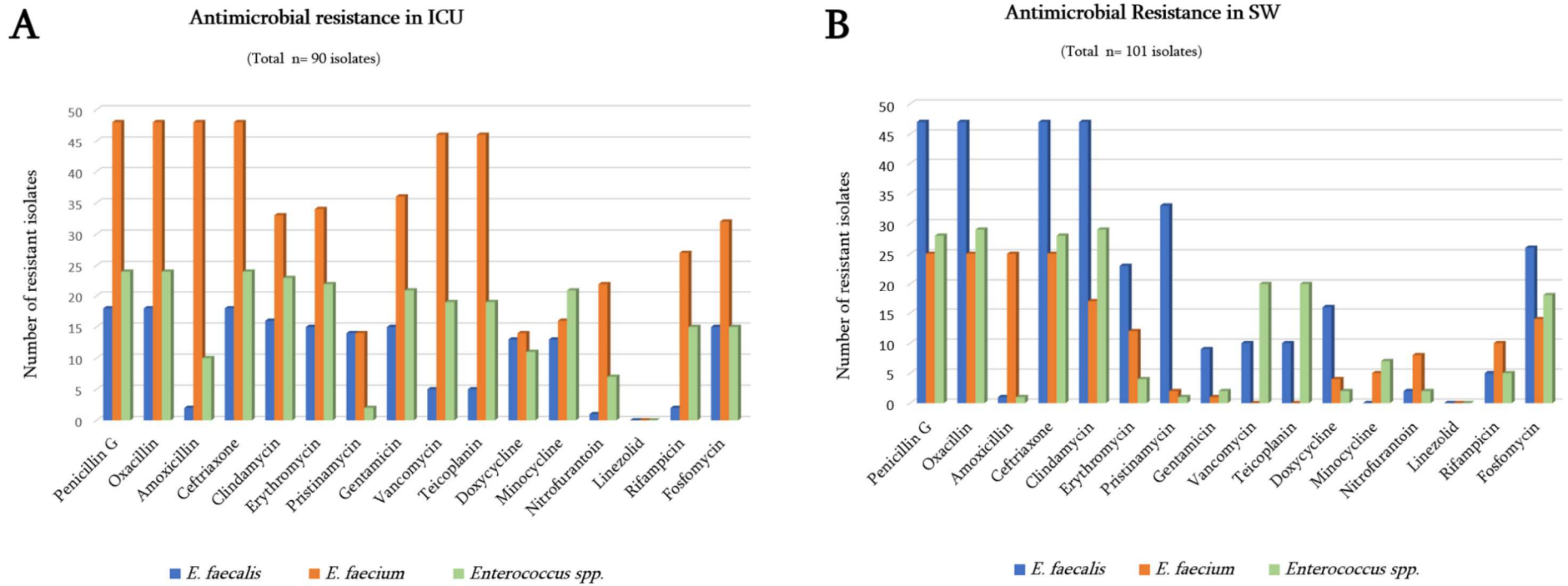
A
Antimicrobial resistance in ICU
(Total n= 90 isolates)
Number of resistant isolates
0
5
10
15
20
25
30
35
40
45
50
Penicillin G
Oxacillin
Amoxicillin
Ceftriaxone
Clindamycin
Erythromycin
Pristinamycin
Gentamicin
Vancomycin
Teicoplanin
Doxycycline
Minocycline
Nitrofurantoin
Linezolid
Rifampicin
Fosfomycin
E. faecalis
E. faecium
Enterococcus spp.
B
Antimicrobial Resistance in SW
(Total n= 101 isolates)
Number of resistant isolates
0
5
10
15
20
25
30
35
40
45
50
Penicillin G
Oxacillin
Amoxicillin
Ceftriaxone
Clindamycin
Erythromycin
Pristinamycin
Gentamicin
Vancomycin
Teicoplanin
Doxycycline
Minocycline
Nitrofurantoin
Linezolid
Rifampicin
Fosfomycin
E. faecalis
E. faecium
Enterococcus spp.

Figure 2.