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Enterococcal species distribution, antibiotic susceptibility and *Van* gene frequency among patients at a tertiary hospital in Sabah

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ABSTRACT

Aims: Enterococcus bacteria, including some strains that are resistant to antibiotics like vancomycin can pose a threat to public health. The purpose of this study is to identify the species, antibiotic susceptibility profile and *VanA*/*VanB* gene frequencies in Enterococci isolated from patients at a tertiary hospital in Kota Kinabalu, Sabah.
Methodology and results: Various bodily fluid specimens were collected from 162 patients between July 2019 and June 2021. Species confirmation and susceptibility testing were performed using an automated system. Subsequently, PCR was used to determine the presence of *VanA and VanB* genes. Species identification revealed the presence of five enterococcal species, namely *E. faecalis* (91), *E. faecium* (64), *E. gallinarum* (3) *E. casseliflavus* (2), along with one isolate each of *E. hirae* and *E. avium*. Overall, resistance to antibiotics like ampicillin, quinolones, tetracycline, gentamicin-syn, nitrofurantoin, glycopeptides and linezolid was generally low (<50%). However, a significant number of isolates displayed high resistance to erythromycin (>50% of samples), while resistance to tetracycline was more moderate. The frequencies of *VanA* and *VanB* genes were low (0.6 and 0%, respectively) and they were only detected in *E. faecium*.
Conclusion, significance and impact of study: The results indicate that while the prevalence of vancomycin-resistant enterococci (VRE) may be low, there is an increasing incidence of multidrug-resistant enterococci, particularly with regards to erythromycin.

Keywords: Antibiotics, *Enterococcus*, *Enterococcus faecalis*, *Enterococcus faecium*, vancomycin resistant

INTRODUCTION

Enterococci are a diverse and adaptable group of Gram-positive bacteria found in humans and animals, with *E. faecalis* and *E. faecium* being the predominant species. These facultative anaerobic bacteria may become a life-threatening nosocomial pathogen, causing endocarditis, sepsis and infections of the skin, urinary and respiratory tract (NNIS System, 1999; Lebreton *et al*., 2014; Dahl *et al*., 2019). They can survive in harsh environments in community and hospital settings due to their inherent and acquired antibiotic resistance mechanisms (Arias and Murray, 2012) and this creates an ongoing source of infection. Their natural resistance to antibiotics (e.g. penicillin, ampicillin and most cephalosporins) and ability to rapidly acquire virulence and multidrug resistance are exacerbated by the abuse and overuse of broad-spectrum antimicrobial, which appears to accelerate the process of colonisation and increases the host susceptibility to diseases. In particular, *E. faecalis* and *E. faecium* are intrinsically resistant to cephalosporins, aminoglycosides, clindamycin, trimethoprim-sulfamethoxazole and fusidic acid. Additionally, *E. faecalis* is also resistant to quinupristin. These enterococci can also acquire additional resistance via horizontal gene transfers (HGT) and mobile genetic elements such as transposons and plasmids (Cetinkaya *et al*., 2000; Eliopoulos and Gold 2001). Plasmids are believed to confer the greatest impact because they can share within and between bacteria species (Jovanovic *et al*., 2017). Although rare enterococcal species like *E. gallinarum* and *E. casseliflavus* are less pathogenic than *E. faecalis* and *E. faecium*, they may also develop resistance towards antibiotics (Reid *et al*., 2001; Eshaghi *et al*., 2015).

Vancomycin, a glycopeptide antibiotic, is one of the frontline drugs used to treat complicated infections, particularly those caused by methicillin-resistant *Staphylococcus aureus* (MRSA). It inhibits peptidoglycan

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synthesis, which in turn, prevents the formation of bacterial cell walls. Besides the misuse and overuse of antibiotics, poor infection prevention control has been identified as a risk factor in the development of vancomycin resistant enterococci (VRE). Managing VRE cases can be challenging because clinicians have limited options. Hence, hospital managements are encouraged to monitor the prevalence of Enterococci species in their patients and screen them for vancomycin resistance (Eliopoulos and Gold, 2001; Reid *et al*., 2001; ECDC, 2020).

Vancomycin resistance may be classified into two types, which all involve the presence of the *Van* gene (Cetinkaya *et al*., 2000; Arias and Murray 2012). The first occurs through the acquisition of *VanA* and *VanB* genes, most commonly in *E. faecium* and *E. faecalis.* The *VanA* and *VanB* genes are transferable and has been reported in *E. faecium*, *E. raffinosus*, *E. avium, E. gallinarum*, *E. casseliflavus* and *E. durans* (Reid *et al*., 2001; Kawalec *et al*., 2007; Rangberg *et al*., 2019). The second intrinsic resistance is found in *E. gallinarum*, *E. casseliflavus* and *E. flavescens*. These species can produce *VanC* enzymes that catalyse the production of D-alanyl-D-serine dipeptides, which can be incorporated into the peptidoglycan pentapeptide precursor. The precursors with these dipeptides residues have low affinity to vancomycin, and thus, their cross-linking and formation of bacterial cell walls will not be affected by the antibiotic (Eliopoulos and Gold, 2001; Reid *et al*., 2001; Kawalec *et al*., 2007).

Hospital administrators have been encouraged to screen their patients for enterococci as different species may show a different pattern to antibiotic resistance. The presence of Enterococci with *VanA* and *VanB* genes indicates a high risk of VRE infection as the genes are highly transferable and extra precaution should be taken by the hospital management to alleviate the risk. The *VanC* gene, on the other hand, is not transferable and has not been linked to VRE outbreaks. The monitoring of antibiotic resistance in hospitals should be prompt and accurate to ensure a safe environment for patients to recover and prevent the spread of nosocomial infection into the community. Moreover, vancomycin resistant bacteria can spread covertly and may not produce noticeable infections right away. Early cases can be detected through screening particularly in healthcare settings, before they progress to larger outbreaks. Early screening is a proactive and preventive measure that enables timely action and controlling the spread of antibiotic-resistant bacteria, even in an area with low or no known cases.

Despite the increasing reports of VRE cases globally, there is a distinct lack of data regarding its frequency and the molecular characterization of VRE isolates, in the Malaysian state of Sabah. The aim of this study is to determine the diversity of enterococcal species and investigate their antibiotic susceptibility profiles and *Van* gene frequencies from clinical samples of Queen Elizabeth Hospital in the state capital of Kota Kinabalu. As the main public healthcare hospital, this hospital receives patients throughout the state, and it is all the more vital to monitor antibiotic resistance to keep the risk of outbreaks as low as possible.

MATERIALS AND METHODS

Clinical, specimens and bacterial isolation

Between July 2019 and June 2021, a total of 162 bodily fluid samples were collected comprising urine (69), blood (54), pus (11), tissue (12), vaginal swabs (4) and other (12) from hospitalized patient in Queen Elizabeth Hospital at Kota Kinabalu, Sabah. These samples were cultured for enterococcal species identification and antibiotic sensitivity test. In the Genomic Laboratory of Universiti Malaysia Sabah, each sample was dipped with an inoculation loop and systematically streaked across plates of selective agar (Hi-Media Laboratories, India) using aseptic techniques. The agar plates were incubated at 37 °C for 24 h with ventilation. Pinkish red colonies that appeared were isolated and streaked on blood agar before incubating at 37 °C for another 24 h. The enterococci colonies would appear white as non- or alpha-haemolytic, and in rare strains, it may be beta-haemolytic with a diameter of 1 mm to 2 mm. A single colony was selected and incubated overnight in LB broth. The broth was centrifuged at 20,000× *g* for 5 min and the pellets were collected for genomic DNA extraction using the Qiagen DNeasy blood and tissue kit (Qiagen Ltd, Valencia, CA, USA) according to the manufacturer's protocols. The morphology of colonies on the blood agar were recorded for initial identification of enterococcal isolates.

Species identification and antimicrobial susceptibility testing

The VITEK 2 system (bioMérieux, Inc., Durham, NC, USA) was used to identify bacterial species and to detect the antibiotic sensitivity profiles by determining the minimum inhibitory concentration (MIC) of each bacterial isolate. The bacterial samples were prepared and Gram-positive identification card (ASP_GP67) was used according to the manufacturer's instructions. The data were recorded and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints.

Species confirmation and *Van* gene screening

Polymerase chain reaction (PCR) and sequencing studies were performed at the Genomic Laboratory of Universiti Malaysia Sabah, Kota Kinabalu. PCR was used to determine the bacterial species and to detect *VanA* and *VanB* genes using published primers listed in Table 1 (Dutka-Malen *et al*., 1995; Kariyama *et al*., 2000). Each reaction mixture comprised of 1× PCR buffer, 2 mM $MgCl_2$, 10 pmol of the respective forward and reverse primers, 0.2 mM dNTP, 1 U of *Taq* DNA polymerase and25 ng of DNA as template, topped up to a final volume of 25 µL with distilled water. Initial denaturation was set at

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Table 1: List of primers used for *Enterococcus* species and *Van* gene identification.

Primer specificity	PCR product (bp)	Primer pair sequences (primer name)	References
E. faecalis	941	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' (ddlE1) 5'-ACGATTCAAAGCTAACTGAATCAGT-3' (ddlE2)	Kariyama *et al*. (2000)
E. faecium	658	5'-TTGAGGCAGACCAGATTGACG-3' (ddlF1) 5'-TATGACAGCGACTCCGATTCC-3' (ddlF2)	Kariyama *et al*. (2000)
16S rDNA	800	5'-GACTACCNGGGTATCTAATCC-3' (804 RX (442) 5'-AGAGTTTGATCCTGGCTNAG-3' (10FX (444)	Dutka-Malen *et al*. (1995)
VanA	1030	5'-CATGAATAGAATAAAAGGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	Kariyama *et al*. (2000)
VanB	433	5'-GTGACAAACCGGAGGCGAGGA-3' 5'-CCGCCATCCTCCTTGCAAAAAA-3'	Kariyama *et al*. (2010)

Table 2: *Enterococcus* species diversity in clinical specimens from Queen Elizabeth Hospital, Kota Kinabalu, Sabah.

Enterococcus species	Clinical specimens (n=162)	Percentage (%)
E. faecalis	91	56.2
E. faecium	64	39.5
E. avium	1	0.6
E. hirae	1	0.6
E. gallinarum	3	1.9
E. casseliflavus	2	1.2

94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 10 sec, annealing at 55 °C for 40 sec and elongation at 72 °C for 30 sec. A final elongation step at 72 °C for 10 min was included. The PCR products were subjected to electrophoresis in 2% agarose gel with a 100 bp DNA ladder as the size standard. The bands were stained with ethidium bromide (0.5 µg/mL) and visualized on a Gel Doc EZ System imager (Bio Rad, Hercules, CA, USA).

The species-specific primer was used for the identification of *E. faecalis* and *E. faecium* species and *Van* gene primer was used for the detection of *VanA* and *VanB* genes. These were confirmed through the appearance of their PCR product bands on the gel electrophoresis. For enterococcal species other than *E. faecalis* and *E. faecium*, sanger sequencing was performed on 16S rDNA amplicons on an ABI 3031 sequencer (Applied Biosystem, Foster City, CA, USA). The amplified sequences of 16S rDNA were compared with those stored in GenBank database using the Basic Local Search Alignment Tool (http://blast.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Frequency of enterococci species

Enterococci were isolated from all 162 bodily fluid samples, and most colonies growing on the blood agar had a diplococcal appearance. Based on colony morphology, PCR and gene sequencing, six enterococcal species were identified as stated in Table 2.

In this study, *E. faecalis* (56.2%) had significantly higher prevalence than *E. faecium* (39.5%) and the other enterococcal species (4.3%), which concurred with a previous study in China and two others in Malaysia (Al-Talib *et al*., 2015; Moussa *et al*., 2019; Zhou *et al*., 2020). However, another two studies in Iran and Ethiopia, found that *E. faecium* had higher prevalence than *E. faecalis* (57.0% vs 35.0% and 35.1% vs 29.8%) in their samples (Abamecha *et al*., 2015; Sattari-Maraji *et al*., 2019). The reason for this disparity could be related to the clinical specimens. Rectal swabs were used in this study, where there was a high count of *E. faecalis*, while the other studies used urine, blood or wound swabs. The high rate of *E. faecalis* colonization was likely due to the ubiquitous nature and predominance of this species in the human intestine (Cetinkaya *et al*., 2000) as well as its mere possession of various virulence and resistance genes (Manson *et al*., 2010). The lower frequency of *E. faecium* and other *Enterococcus* species in this study corresponded with global reports, which stated that 80-90% of enterococcal infections in human could be attributed to *E. faecalis*, 10-15% to *E. faecium* and 5% to other species combined (Cetinkaya *et al*., 2000; Eliopoulos and Gold, 2001).

E. gallinarum was the third-most common *Enterococcus* species, accounting for 1.9% of all samples, which was consistent with reports that stated this species as responsible for 2% of hospital-acquired infections (Reid *et al*., 2001; Britt and Potter, 2016). Despite the fact that *E. gallinarum* was rarely isolated from clinical specimens, particularly in developed countries, several reports had implicated it as the cause of serious infections in the abdomen, pelvis, biliary tract and wounds (Reid *et al*., 2001; Britt and Potter, 2016; Mastor *et al*., 2020). Furthermore, a few reports had highlighted other enterococcal species containing *VanA* and *VanB* genes with a high level of vancomycin resistance (Schooneveldt *et al*., 2000; Mammina *et al*.,

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Table 3: Overall antimicrobial susceptibilities profiles of enterococcal isolates investigated.

Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Erythromycin	96 (59.5)	11 (6.7)	55 (34)
Tetracycline	80 (49.4)	8 (4.9)	74 (45.7)
Ciprofloxacin	63(38.9)	2 (1.2)	97 (59.9)
Ampicillin	63(38.9)	0	99 (61.1)
Gentamycin-syn	37 (22.8)	1 (0.7)	124 (76.5)
Nitrofurantoin	2	3 (1.9)	157 (96.9)
Vancomycin	1	0	162 (100)
Teicoplanin	0	0	161 (99.4)
Linezolid	3	6 (3.7)	153 (94.4)
Daptomycin	0	0	162 (100)

2005), suggesting that motile enterococci may contribute to the spread of vancomycin resistance. Similarly, *E. avium* and *E. hirae* were rarely reported in nosocomial infections. This study only identified one each of *E. hirae* (JAKEIR000000000), *E. avium* (JAKEIQ000000000) and *E. casseliflavus* species. The *Enterococcus* species discovered in this study had been implicated in disease and this affirmed the diverse species that could present in nosocomial infections. However, this study involved patients at one hospital only, thus the results should not be used to draw conclusions about *Enterococcus* colonization in the population as a whole. In the United States, the clinical and epidemiological features of bacteremia caused by *E. gallinarum* and *E. casseliflavus* had been reported in up to 95% of patients compromised by immunodeficiency and malignancy (Reid *et al*., 2001; Britt and Potter, 2016). The frequency of *E. gallinarum* in this study was in agreement with a previous study by Ruoff *et al*. (1990) (1%).

Antibiotic susceptibility profiles and *Van* gene frequencies

Antibiotic resistance was generally low (<50% of samples) or all the species especially ampicillin, quinolones (ciprofloxacin), gentamicin-syn, nitrofurantoin, glycopeptides, linezolid and daptomycin. However, it seemed to be quite high for erythromycin and fairly distributed for tetracycline (Table 3). *E. faecalis* isolates were 100% susceptible to vancomycin, teicoplanin and daptomycin but resistant to erythromycin (56%) and tetracycline (57.1%). Of the three isolates that were resistant to linezolid, two were E. faecalis species (Table 4). For *E. faecium*, all isolates were 100% susceptible to teicoplanin and daptomycin. However, two were non-susceptible to nitrofurantoin and one isolate showed resistance to vancomycin. This study also found *E. faecium* exhibiting resistance to multiple antibiotics such as erythromycin (68.8%), ciprofloxacin (73.4%) and ampicillin (76.6%) compared with *E. faecalis* species which showed high resistance to erythromycin (56.0%) and tetracycline (57.1%) only (Table 4). Thus, it could be deduced that the majority of multidrug resistance MDR isolates belonged to *E. faecium*. For rare enterococcal species, only *E. gallinarum* showed resistance to more than one antibiotic: erythromycin, ciprofloxacin, ampicillin and gentamicin-syn (Table 5).

Almost all the enterococci in this study were susceptible to at least 94% of the last-line antibiotics tested, including nitrofurantoin (96.9%), vancomycin (100%), teicoplanin (99.4%), linezolid (94.4%) and daptomycin (100%). In terms of species, *E. faecium* showed a higher percentage of resistance than *E. faecalis*, which was consistent with the findings of Sattari-Maraji *et al*. (2019). As ampicillin was the drug of choice in treating of enterococcal infections, the significantly low susceptibility of isolates especially in *E. faecalis* implied that the usage of ampicillin along with other antibiotics such as ciprofloxacin, gentamicin and nitrofurantoin could still be the drug of choice and was effective for enterococcal treatment in the hospital. But it should be noted that ampicillin resistance in this study was already high (85.7%) in *E. faecium*, which was consistent with other studies (Cercrenado, 2011; Arias and Murray, 2012; Coombs *et al*., 2013). This could be due to differences in methods for antibiotic susceptibility testing. The use of automated instruments such as in this study was considered generally more efficient compared with the disk diffusion and broth microdilution methods used by other investigators. At the moment, vancomycin resistance in enterococci may not be a cause for concern in Queen Elizabeth Hospital and Sabah in general, but continuous surveillance in hospital settings might be necessary to monitor the trend.

***Van* gene frequencies and multidrug resistant phenotype**

A vancomycin screening test was performed for all *Enterococcus* isolates using *VanA and VanB* gene specific primer. There was only one *VanA* gene detected from a blood specimen and the bacteria species was identified as *E. faecium* (Figure 1). The *VanA* gene was amplified at 1,030 bp, confirming the VITEK 2 results of *VanA* presence in the isolate. There was no detection of the *VanB* gene in any of the specimens. The resistance rate in other Enterococci revealed that only five isolates showed resistance pattern to either one antibiotic (i.e: erythromycin, ciprofloxacin, ampicillin, gentamicin-syn or linezolid). Meanwhile, more than 71% rare clinical

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Table 4: Percentage and antimicrobial susceptibilities profiles of enterococcal isolates.

Antibiotics	*E. faecalis*, n = 91			*E. faecium*, n = 64			Others n = 7		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Erythromycin	51 (56.0)	5 (5.5)	35 (38.5)	44 (68.8)	4 (6.3)	16 (25)	1 (14.2)	2 (28.6)	4 (57.1)
Tetracycline	52 (57.1)	3 (3.3)	36 (39.6)	28 (43.8)	5 (7.8)	31 (48.4)	0	0	7 (100)
Ciprofloxacin	15 (16.5)	0	76 (83.5)	47 (73.4)	2 (3.1)	15 (23.4)	1 (14.2)	0	6 (85.7)
Ampicillin	13 (14.3)	0	78 (85.7)	49 (76.6)	0	15 (23.4)	1 (14.2)	0	6 (85.7)
Gentamycin-syn	17 (18.7)	0	74 (81.3)	19 (29.7)	0	45 (70.3)	1 (14.2)	1 (14.3)	5 (71.5)
Nitrofurantoin	0	1 (1.1)	90 (98.9)	2 (3.1)	2 (3.1)	60 (95.3)	0	0	7 (100)
Vancomycin	0	0	91 (100)	1 (1.6)	0	63 (98.4)	0	0	7 (100)
Teicoplanin	0	0	91 (100)	0	0	64 (100)	0	0	7 (100)
Linezolid	2 (2.2)	3 (3.3)	86 (94.5)	0	2 (3.1)	62 (96.9)	1 (14.2)	1 (14.3)	5 (71.5)
Daptomycin	0	0	91 (100)	0	0	64 (100)	0	0	7 (100)

S: Susceptible, I: Intermediate, R: Resistant, -: Negative; MDR, multidrug resistant (isolate resistant to two or more antibiotic classes); “Others” denotes *E. gallinarum*, *E. avium*, *E. hirae*, *E. casseliflavus*.

Table 5: Antimicrobial profiles of rare enterococcal species.

Antibiotics	*E. gallinarum* n = 3			*E. casseliflavus* n = 2			*E. hirae* n = 1			*E. avium* n = 1			No. of resistant isolates
	R	I	S	R	I	S	R	I	S	R	I	S	
Erythromycin	1		2			2		1			1		1
Tetracycline			3			2			1			1	0
Ciprofloxacin	1		2			2			1			1	1
Ampicillin	1		2			2			1			1	1
Gentamycin-syn	1		2		1	1			1			1	1
Nitrofurantoin			3			2			1			1	0
Vancomycin			3			2			1			1	0
Teicoplanin			3			2			1			1	0
Linezolid			3			2			1		1		0
Daptomycin			3			2			1			1	0

*n = number of enterococcal isolates; R: Resistant; I: Intermediate; S: Susceptible.

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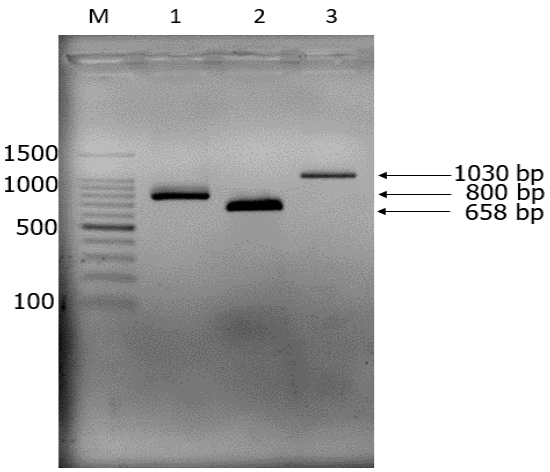

Figure 1: PCR amplification of vancomycin resistance *Enterococcus* isolates using species specific and *Van* gene primers in a patient sample. Lane M: 100 bp DNA Ladder (TransGen Biotech, China); Lane 1: 16S rDNA (800 bp); Lane 2: *E. faecium* (658 bp); Lane 3: *VanA* resistant gene (1,030 bp). The GenBank accession number for vancomycin resistance *E. faecium* in this study is JAOTGP000000000.

enterococcal species showed resistance to ciprofloxacin (85.7%), ampicillin (85.7%), gentamicin (71.5%) and linezolid (71. 5%).

The frequency of *Van* genes in this study was much lower than the results obtained by two other studies (0.1% and 2.9%) in Peninsular Malaysia (Ibrahim *et al*., 2011; Mohamed *et al*., 2015). However, Weng *et al*. (2013) and Daniel *et al*. (2017) reported no VRE isolates in their Malaysian clinical isolates. Worldwide, the prevalence of antibiotic-resistant enterococcal infections is rising (Daniel *et al*., 2017; Shiadeh *et al*., 2019). Several studies reported that the *VanA* genotype was of serious concern due to the risk of transmission to other organisms and it was more common than *VanB* (Azzam *et al*., 2023). From an epidemiological point of view, the most dangerous VRE were the *VanA* and *VanB* genotypes as they were responsible for the majority of acquired transferable resistance (Mira *et al*., 2014). The contrasting geographic burden of disease imposed by VRE across select countries, on the other hand, had been shown to correlate with national antimicrobial stewardship and surveillance practices.

This study has several limitations. Firstly, the clinical specimens were collected solely from one tertiary hospital that adheres to well-established hospital infection control guidelines. This may limit the generalizability of the findings to other healthcare settings. Another limitation pertains to the use of the VITEK 2 system. While this automated system was expected to provide accurate results, it failed to confirm the species of seven isolates. However, this issue could be addressed through the utilization of 16S rDNA sequencing and subsequent comparison with the GenBank database, offering a potential resolution.

CONCLUSION

As a conclusion, the antibiotic-resistant patterns of the clinical *Enterococcus* isolates suggest low resistance to vancomycin at Queen Elizabeth Hospital. However, most of the enterococci species have developed resistance against erythromycin. A multidrug resistance (MDR) trend is seen in *E. faecium*, with resistance developed against erythromycin, ampicillin and ciprofloxacin. Thus, continuous surveillance of antimicrobial susceptibility may be necessary for optimal antibiotic usage and control of antibiotic bacteria at the hospital, especially when it involves cases of *E. faecium*.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research was approved by the Medical Research Ethics Committee (MREC), Ministry of Health, Malaysia (No. NMRR-19-1770–48622) and Universiti Malaysia Sabah Medical Ethics Committee (No. JKEtika 1/19(26).

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