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Distribution of multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens in the Zainoel Abidin General Hospital, Banda Aceh, Indonesia

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Abstract. *Hayati Z, Desfiana UH, Suhartono S. 2022. Distribution of multidrug-resistant* Enterococcus faecalis *and* Enterococcus faecium *isolated from clinical specimens in the Zainoel Abidin General Hospital, Banda Aceh, Indonesia. Biodiversitas 23: 5043-5049. Enterococcus faecalis* and *Enterococcus faecium* are two predominant enterococcal species associated with opportunistic infections, especially in clinical settings. This study aimed to determine the distribution and antibiotic sensitivity of *E. faecalis* and *E. faecium* isolated from clinical specimens in the Zainoel Abidin General Hospital during 2019-2022. This study involved isolation, identification, and testing of antibiotic susceptibility of *E. faecalis* and *E. faecium* isolates. A total number of 299 enterococcal isolates detected in this study consisting of 233 (7.92%) isolates of *E. faecalis* and 66 (2.18%) isolates of *E. faecium*. Both *E. faecalis* and *E. faecium* shared phenotypic features in common. *Enterococcus faecalis* isolates exhibited less sensitivity to benzathine penicillin G, ciprofloxacin, levofloxacin, tetracycline, high-level streptomycin, doxycycline, and fosfomycin. *Enterococcus faecium* had less sensitive to all tested antibiotics, except for linezolid, vancomycin, and tigecycline. Based on the clinical specimens, *enterococci* were predominantly found in urine specimens accounting for 42.80%, while based on patient's age, enterococci were prevalently found in patients aged >46 years accounting for 72%. The distribution of both enterococci was evenly detected in both male and female patients. Based on the wards, enterococci were predominantly detected (50.65%) in patients treated in the internal medicine wards. Overall, the present study suggested the susceptibility of enterococcal pathogens and determined the most effective antimicrobials as empirical therapy to treat the infections. The surveillance programs and infections control should be effectively implemented to manage these multidrug-resistant enterococcal infections particularly within healthcare settings.

Keywords: Antibiotic susceptibility, clinical specimens, *Enterococcus faecalis*, *Enterococcus faecium*, Multidrug-resistant

INTRODUCTION

Bacterial infections remain a major concern due to their significant implications in communal and hospital settings. One group of bacteria associated with opportunistic infections, particularly in healthcare settings is enterococci. Enterococci-associated infections might range from urinary tract infections, bacteremia, and endocarditis, wound infections to intra-abdominal infections. Enterococcal infections have been increasing concern in recent years as the infections generate considerable consequences on mortality, morbidity, and economic impacts. It was estimated that the enterococcal infections, particularly pathogens that are vancomysin resistant, increased the odds of mortality, major surgical procedure, admission to intensive care unit, length of hospital stays, and discharge to a long-term care facility by 1.4 - 3.5 folds (Carmeli et al. 2002).

Enterococci are Gram-positive non-spore-forming cocci that formin pairs or as a short chain that are facultative anaerobes and non-motile. Moreover, enterococci are catalase-negative and able to grow at media containing 6.5% salt with pH 4.8-9.6 and optimal temperature of 35-37^{o}C (Ch'ng et al. 2019). Enterococci are commonly found in nature and as well in the human and animal gastrointestinal tracts as commensal. Enterococci are currently the leading causative agents for hospital-acquired infections. Moreover, it is well known that enterococci survive on living and inanimate surfaces for extended periods of time, serving as reservoirs for the spread of infections in hospitals. The pathogenesis of enterococcal infections worsen in critically ill and immunocompromised patients who have had prolonged antibiotic treatment and hospitalization. This underlying medical or immune condition of the patients who have been commonly exposed to previous infections and excessive antibiotic administration, as well as previous invasive medical procedures during hospitalization which are the primary risk factors for enterococcal infections (Kajihara et al. 2015; Risqiyah et al. 2022). In addition to critically ill patients, enterococci are also currently identified as etiological agents in patients suffering from COVID-19 infections. The SARS-CoV-2 infections appear to have a synergistic effect on enterococcal infections *via* modifications in the bacterial microbiome, favoring enterococci and increasing intestinal permeability, creating

the ideal environment for enterococci to cause invasive infections (Toc et al. 2022).

The increasing number of enterococcal infections might be related to the virulence factors of the pathogens as well as the development of antibiotic resistance and the formation of biofilm structures. Enterococci possess some virulence factors that facilitate the bacterial pathogen attaching to and colonizing the host cell causing disease and evade the host immune system. Enterococcal virulence factors include extracellular surface proteins (ESP); gelatinase; and hemolysins allowing enterococci to attach to the host cells; cleave fibrin and damage host tissues; and hydrolyze collagen and gelatin forming pores in the host cytoplasmic membrane (Ben Braïek and Smaoui 2019). In terms of antibiotic resistance, enterococci are identified for their ability to develop resistance to a variety of antimicrobials ranging from ampicillin, cephalosporin, aminoglycosides, and vancomycin to the newer antibiotics such as daptomycin and oxazolidinones (Miller et al. 2020). This development of resistance is owed to the combination of both intrinsic and acquired antibiotic resistance genes through mutation or lateral gene transfer (Chajęcka-Wierzchowska et al. 2019). An additional factor contributing to the increasing tolerance to antibiotics of enterococci is the formation of biofilm. Biofilm structures allow the enterococci to be more tolerant to antibiotics by lowering the drug penetration leading to persistent infections (Ch'ng et al. 2019).

Among enterococci, there are a variety of enterococcal species harbored in human gastrointestinal tracts, namely *Enterococcus avium, E. casseliflavus, E. durans, E. gallinarum, E. hirae, E. mundtii, E. raffinosus, E. faecalis,* and *E. faecium* (García-Solache and Rice 2019). *Enterococcus faecalis* and *Enterococcus faecium* are the two most prominent and clinically relevant enterococcal species. The former is responsible for 85-95% of nosocomial infections, whereas the latter species involves in 5-10% (Moghimbeigi et al. 2018). Both *E. faecalis* and *E. faecium* are comparable in genetic and ecological aspects as the first is less frequently involved in developing multidrug-resistant and spreading antibiotic resistance than the latter (Cattoir 2022).

Zainoel Abidin General Hospital in Banda Aceh, Indonesia is the primary regional hospital admitting patients suffering from various infectious diseases including those associated with enterococcal infections, in Aceh. The excessive and inappropriate use of antibiotics may raise concern for development of multidrug-resistant enterococci associated with increasing cases of infections in the region. Hence, the present study aimed to evaluate and determine the antibiotic susceptibility and prevalence of multidrug-resistant (MDR) two predominate enterococcal species, namely *E. faecalis* and *E. faecium* based on clinical specimens, and patient's gender, age, and hospital wards in the Zainoel Abidin general hospital Banda Aceh, Indonesia. To the best of our knowledge, this is the most current study comparing the occurrence of MDR *E. faecalis* and *E. faecium* isolates among clinical specimens in the region during the study period.

MATERIALS AND METHODS

Clinical sample collections

Blood, urine, sputum, body fluid, pus, and swabs were collected from inpatients and outpatients at the Zainoel Abdin general hospital in Banda Aceh, Indonesia, from March 2019 to March 2022 for this study. All clinical samples were evaluated for quality and the sample identity, i.e., types of clinical samples, age, gender, and the hospital wards of the patients were documented. The ethical clearance committee for health research, Faculty of Medicine, Universitas Syiah Kuala (registration number 386/EA/FK-RSUDZA/2021) has authorized the research.

Bacterial isolation and identification

All clinical samples were inoculated onto blood agar (Merck, Germany) and MacConkey agar (Merck, Germany) plates, except for blood samples which were pre-cultured using BacT/ALERT® 3D (Biomeriux, Lyon, France) according to the manufacturer's instructions. The plates were then incubated for 24 hrs at 37C before being Gram-stained and inspected for morphological characteristics under a microscope with 1000x magnification. VITEK®2 Compact (Biomeriux, Lyon, France) was used for further identification as well as antibiotic susceptibility assay. A pure bacterial colony isolated from clinical samples was suspended in 0.45 % NaCl corresponding to 1.8-2.2 McFarland Standard solution prior to inoculation into cassettes for identification and antibiotic susceptibility, respectively. Antibiotic susceptibility determination was performed following the guidelines of Clinical and Laboratory standards (CLSI) using antibiotics as follows: benzathine penicillin G, amoxicillin, ampicillin, amoxicillin-clavulanate, ampicillin/ sulbactam, ciprofloxacin, levofloxacin, piperacillin tazobactam, linezolid, vancomycin, doxycycline, quinupristin/ dalfopristin, tetracycline, tigecycline, nitrofurantoin, and fosfomycin.

Statistical analysis

The isolated bacteria from the clinical specimens were descriptively analyzed for their distribution based on clinical specimens, and patient's gender, age, and hospital wards. All data was tabulated using Microsoft Excel to generate descriptive information in tables or charts. A statistical analysis was performed using Chi-square test or Fisher's exact test when appropriate. All tests performed using XLStat cloud (Addinsoft, New York, USA) were considered statistically significant at a $p \leq 0.05$ on two-tailed.

RESULTS AND DISCUSSION

In the present study, there were 233 isolates of *E. faecalis* and 66 isolates of *E. faecium* in total isolated from clinical specimens with prevalence of occurrence 1.10% and 0.31%, respectively (out of total of 21,115 clinical samples submitted for analysis (data not shown)) during the study period of March 2019 to March 2022.

Macroscopically, both colony morphology of *E. faecalis* (Figure 1A) and *E. faecium* (Figure 1B) shared phenotypic features in common: were round, white, smooth edges; convex elevation; and non-hemolytic colonies on blood agar. Likewise, both microscopic observations at 1,000x magnification showed that *E. faecalis* (Figure 1C) and *E. faecium* (Figure 1D) exhibited typical Gram-positive cocci in pairs (diplococci) or short chains.

Enterococcus faecalis and *E. faecium* isolates had quite different antibiotics susceptibility (Table 1). *Enterococcus faecalis* isolates remain moderately sensitive to glycopeptide group, namely vancomycin, which was 73% of a total 233 isolates. The moderate sensitivity of the isolates was also for tigecycline, amoxicillin and linezolid, ampicillin, amoxicillin-clavulanate, ampicillin/sulbactam, nitrofurantoin, and piperacillin tazobactam. Meanwhile, *E. faecalis* isolates exhibited less sensitivity to benzathine penicillin G, ciprofloxacin, levofloxacin, tetracycline, high-level streptomycin, doxycycline, and fosfomycin. Contrary to the counter part, *E. faecium* had less sensitive to all tested antibiotics, except for linezolid, vancomycin, and tigecycline. Based on this antibiotic susceptibility, both *E. faecalis* and *E. faecium* isolates can be classified as multidrug resistance since the isolates demonstrated their resistance to more than antimicrobial classes in the present study.

The results of enterococcal distribution based on types of clinical specimens, patient's gender, age, and hospital wards in the Zainoel Abidin General Hospital were intriguing. During the three-year study period, enterococcal infections were discovered most frequently in urine samples, which accounted for nearly half of all enterococcal-positive clinical samples in this investigation (Figure 2). The enterococcal infections were also detected in pus and blood samples accounting for 20.06% and 18.72%, respectively. Furthermore, *E. faecalis* and *E. faecium* isolates showed different distributions based on clinical specimens, i.e., *E. faecalis* were more frequently detected in all clinical specimens in this study compared to *E. faecium.* The distribution of *E. faecalis* and *E. faecium* based on the types of clinical specimen was statistically independent (P = 0.067; χ^2 = 11.79), i.e., there was no association in the distribution between enterococcal species and clinical specimens.

In terms of patient's age groups (Figure 3), it can be seen that while *E. faecalis* was detected in patients regardless of age, *E. faecium* was collectively detected in patients older than 46 years old and was absent from those aged 12-16 years. Similarly, the distribution of *E. faecalis* and *E. faecium* based on the patient's age groups was also statistically independent (P = 0.287; χ^2 = 9.692), i.e., there was no association between enterococcal species and age groups. Furthermore, the distribution of enterococci was evenly detected in both male and female patients (Figure 4). *Enterococcus faecalis* were more frequently detected in both genders, i.e., for 82% in males and 74% in females, in this study compared to *E. faecium* accounting for 18% in males and 26% in females. Again, the distribution of *E. faecalis* and *E. faecium* based on the patient's gender in the present study was also statistically independent (P = 0.100; χ^2 = 2.710), i.e., there was no association on the distribution between enterococcal species and patient's gender.

Enterococcal infections had a different distribution based on the hospital wards. Enterococcal-positive infections were the most frequently detected on the clinical specimens collected from patients being treated in the internal medicine room compared to patients in other wards accounting for 116 isolates (50.65%) (Figure 5). *Enterococcus faecalis* were more frequently detected in patients in all hospital wards in this study compared to *E. faecium* which were non-detectable in patients being treated in delivery room, tuberculosis (TB) room COVID ICU, cardiac ICU, RHCU operating theatre, emergency room, and intermediate room. The distribution of *E. faecalis* and *E. faecium* based on the patient's wards in the present study was also statistically independent (P = 0.272; χ^2 = 23.345) meaning there was no association in the distribution between enterococcal species and patient's wards.

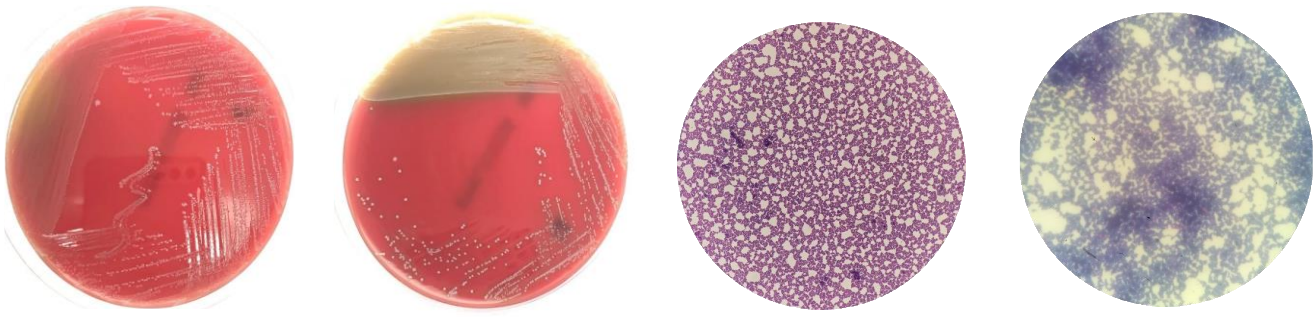

Figure 1. Representation of colony morphology grown onto blood agar media after 24 hrs of (A) *Enterococcus faecalis*; (B) *Enterococcus faecium* isolates and microscopic observation at 1,000x magnification of Gram stained; (C) *Enterococcus faecalis*; (D) *Enterococcus faecium* isolated from clinical specimens

Table 1. Antibiotic susceptibility of *Enterococcus faecalis* (n = 233) and *E. faecium* (n=66) isolates of clinical specimens

Antibiotics	*Enterococcus faecalis (n = 233)*		*Enterococcus faecium (n=66)*		Chi-2[a]	*p-values*[b]
	n	%	n	%		
Benzathine penicillin G	84	36.05	2	3.03	36.252	<0.0001
Amoxicillin	207	88.84	9	13.64	137.624	<0.0001
Ampicillin	206	88.41	9	13.64	135.413	<0.0001
Amoxicillin-clavulanate	207	88.84	9	13.64	137.624	<0.0001
Ampicillin/sulbactam	192	82.40	8	12.12	114.160	<0.0001
Ciprofloxacin	68	29.18	8	12.12	8.874	0.003
Levofloxacin	75	32.19	10	15.15	8.053	0.005
Piperacillin tazobactam	173	74.25	6	9.09	96.747	<0.0001
Linezolid	207	88.84	61	92.42	0.758	0.384
Vancomycin	214	91.85	57	86.36	1.679	0.195
Doxycycline	23	9.87	26	39.39	28.067	<0.0001
Quinupristin/dalfopristin	6	2.58	24	36.36	52.560	<0.0001
Tetracycline	38	16.31	28	42.42	18.415	<0.0001
Tigecycline	212	90.99	62	93.94	0.626	0.429
Nitrofurantoin	187	80.26	12	18.18	86.995	<0.0001
Fosfomycin	8	3.43	4	6.06	0.838	0.360

Note: [a] Chi-square calculated for comparison of susceptibility in *Enterococcus faecalis* versus *Enterococcus faecium*; [b] P values generated from the chi-square

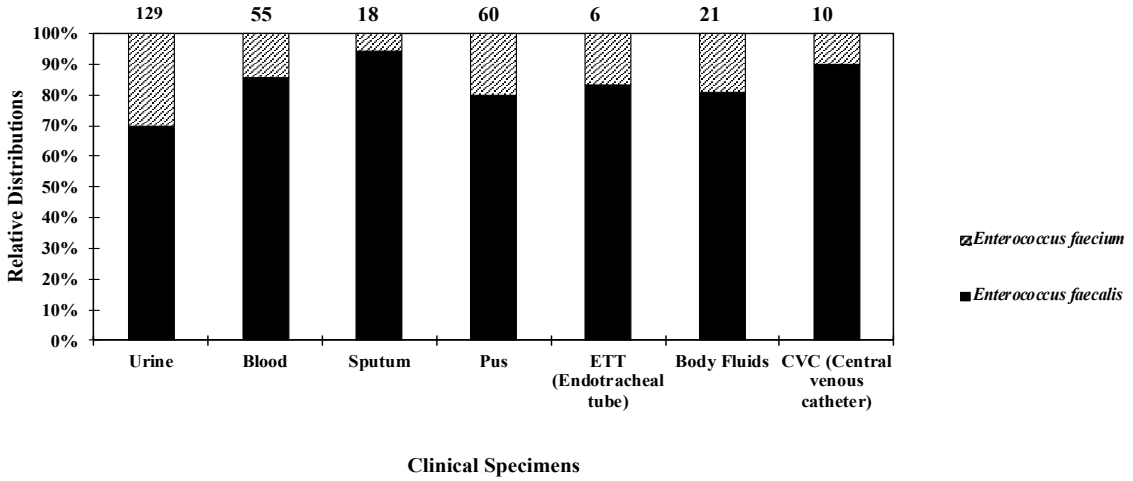

Figure 2. Distribution and frequency of occurrence (%) of *Enterococcus faecalis* (n = 233) and *Enterococcus faecium* (n = 66) isolates based on types of clinical specimens. Numbers above each column are the total number of isolates. Based on the Chi-square test for independence test, types of clinical specimens and enterococcal species were independent ($P = 0.067$; $\chi^2 = 11.79$)

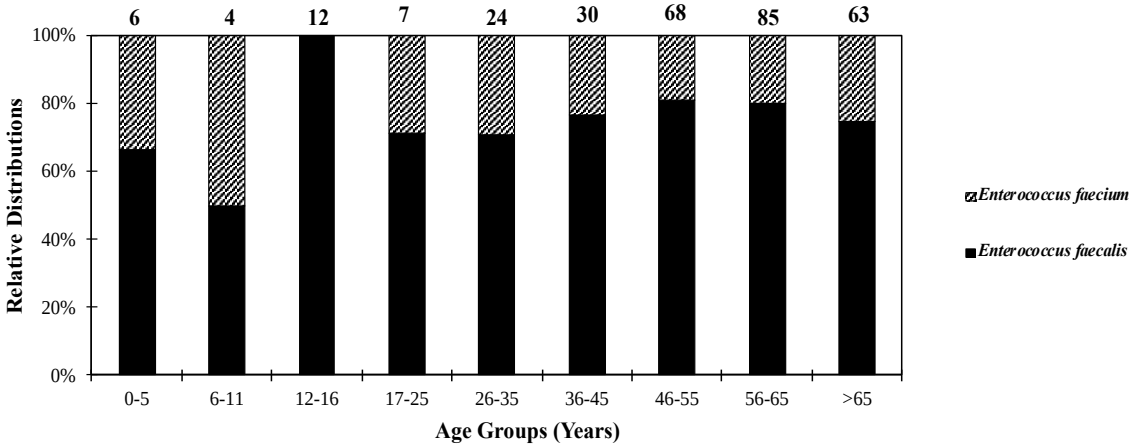

Figure 3. Distribution and frequency of occurrence (%) of *Enterococcus faecalis* (n = 233) and *Enterococcus faecium* (n = 66) isolates based on age groups of patients. Numbers above each column are the total number of isolates. Based on the Chi-square test for independence test, types of clinical specimens and enterococcal species were independent ($P = 0.287$; $\chi^2 = 9.692$)

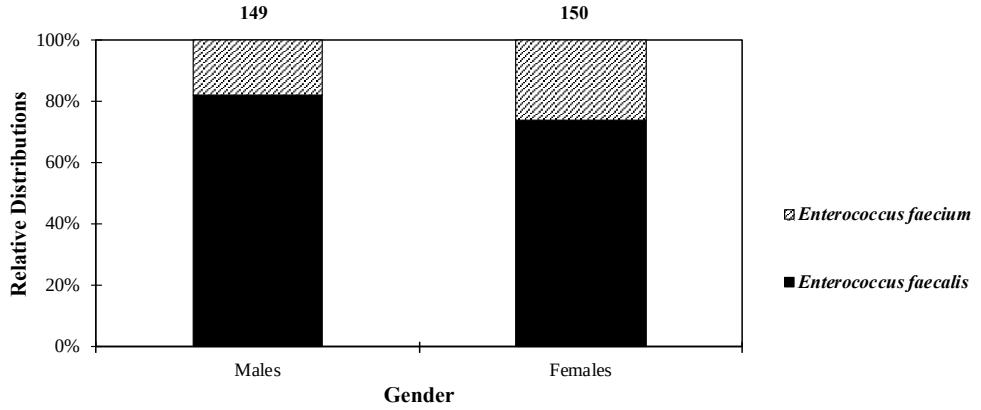

Figure 4. Distribution and frequency of occurrence (%) of *Enterococcus faecalis* (n = 233) and *Enterococcus faecium* (n = 66) isolates based on gender of patients. Numbers above each column are the total number of isolates. Based on the Chi-square test for independence test, types of clinical specimens and enterococcal species were independent ($P = 0.100$; $\chi^2 = 2.710$)

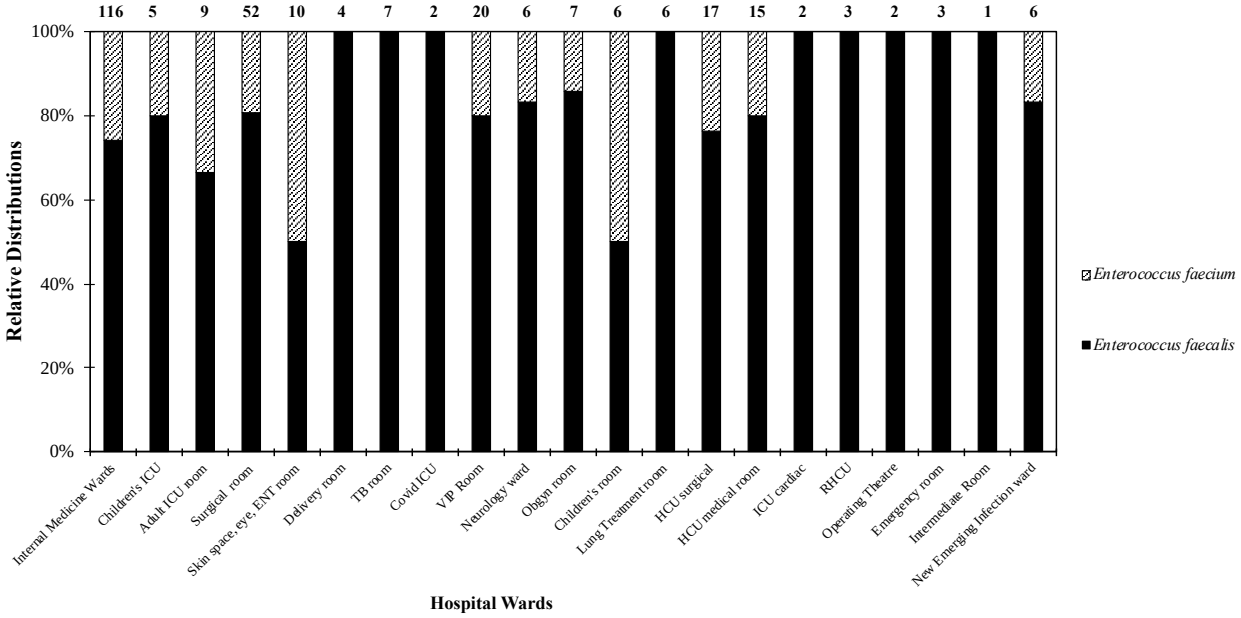

Figure 5. Distribution and frequency of occurrence (%) of *Enterococcus faecalis* (n = 233) and *Enterococcus faecium* (n = 66) isolates based on patients. Numbers above each column are the total number of isolates. Based on the Chi-square test for independence test, types of clinical specimens and enterococcal species were independent ($P = 0.272$; $\chi^2 = 23.345$)

Discussion

Enterococci have been identified as one of the most prominent infection-causing bacteria due to their genome plasticity and metabolic adaptability that enable them to exist in various conditions in environmental and hospital settings (Cattoir 2022). To date, among *Enterococcus* species, *E. faecalis* and *E. faecium* are the two most leading etiological agent for enterococcal infections in healthcare facilities (Moghimbeigi et al. 2018). A total of 299 enterococcal isolates have been recovered in the present study with *E. faecalis* being more prevalent than *E. faecium.* These findings corroborate other previous investigations (Billington et al. 2014; Boccella et al. 2021; Suhartono et al. 2021) signifying the higher species distribution of *E. faecalis* over *E. faecium.* Additionally, the present study was conducted to elucidate the antibiotic susceptibility and prevalence of MDR *E. faecalis* and *E. faecium*, based on clinical specimens, and patient's gender, age, and hospital wards at the hospital.

Morphological observations of both *E. faecalis* and *E. faecium* shared numerous phenotypic determinants in common. Colony morphology of both enterococcal species showed smooth, gray, non-hemolytic translucent colonies on blood agar. Furthermore, microscopic observation showed that both isolates had similarity in cellular arrangement typical of Gram-positive diplococci and short chains. Both *E. faecalis* and *E. faecium* are difficult to differentiate using morphological identification, but they can be identified based on biochemical and molecular approaches. Biochemically, *E. faecalis* are characterized as

a sorbitol and pyruvate fermenter yet non-arabinose fermenter, whereas *E. faecium* has the opposite characteristics (Manero and Blanch 1999). Others also added antibiotic susceptibility as a key characteristic to differentiate both enterococcal species, i.e., *E. faecalis* were susceptible to ampicillin, whereas *E. faecium* was resistant (Quiloan et al. 2012), or using chromogenic-based agar i.e., *E. faecalis* (green colonies) and *E. faecium* (mauve colonies) (Kallstrom et al. 2010). Molecular approaches. i.e., PCR targetting $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ has been utilized nowadays to rapidly and pricisey identify both enterococcal species (Mustafa et al. 2021). The potential benefits of molecular-based approaches over conventional culture and phenotypic testing include better diagnostic accuracy, higher reliability, knowledge about antibiotic resistance, and reduced time and cost (García-Solache and Rice 2019; Sanderson et al. 2019).

The sensitivity of multidrug-resistant *E. faecalis* and *E. faecium* to antibiotics was considerably distinct. *E. faecalis* isolates were highly resistant to benzathine penicillin G, ciprofloxacin, levofloxacin, tetracycline, high-level streptomycin, doxycycline, gentamicin, and fosfomycin. Multidrug-resistant *E. faecium*, on the other hand, was resistant to all tested antibiotics, except for linezolid, vancomycin, and tigecycline. A previous study found the similar result signifying *E. faecium* was resistant to ampicillin, ampicillin/sulbactam, and imipenem accounting for 84.5%, 82.7%, and 86.7%, respectively, and *E. faecalis* was resistant to gentamicin and streptomycin high-level (Boccella et al. 2021). Moreover, the present study indicates that both enterococcal species remain sensitive to linezolid, vancomycin, and tigecycline. These findings corroborate previous report elucidating that both multidrug-resistant enterococcal pathogens remain sensitive to these last resort antibiotics including oxazolidinones (linezolid, tedizolid), novel tetracyclines (tigecycline), and lipopeptides (daptomycin) (Bender et al. 2018). However, it is worth noting that the resistance development of enterococcal pathogens against vancomycin (Shrestha et al. 2021), linezolid (Bi et al. 2018), and tigecycline (Bai et al. 2022) is emerging. The primary mechanism of vancomycin resistance in enterococci is the modification of the peptidoglycan production pathway, which results in decreased binding affinity of vancomycin medicines to the normal cell wall (Ahmed and Baptiste 2017), whereas linezolid resistance is achieved by the mutation of 23S rRNA of ribosomal methyl transferase gene *cfr* as well as *optrA* (oxazolidinone phenicol transferable resistance) gene (Bi et al. 2018), and tigecycline resistance was established through mutation of tigecycline target sites and upregulation of efflux pumps (Bai et al. 2022).

The present study demonstrated the prevalence of enterococci as the primary causative agents for urinary tract infections followed by bloodstream infections since the pathogens were most predominantly detected on urine as well as blood and pus samples. Previous investigation found that enterococci were detected in ten percent out of 791 hospitalized patients diagnosed as complicated urinary tract infections (Turjeman et al. 2021). Additionally, our previous research also found that both *E. faecalis* and *E. faecium* as the major Gram positive uropathogens for about 58% in total of all non-Enterobacteriaceae (n = 107) detected in urine samples of patients suffering from urinary tract infections during a period of February to December 2020 at the Zainoel Abidin Hospital (ZAH) Banda Aceh, Indonesia (Suhartono et al. 2021). In addition as uropathogens, the present study also highlights the pathogens associated with bloodstream and wound infections which are in alignment with previous studies (Jabbari Shiadeh et al. 2019).

Regarding to the patient's age, the present study demonstrated that enterococcal infections prevalently occurred on patients aged 46 years or older. These results are in accordance with other investigations, signifying the prevalence of enterococcal infections on patients aged 55-75 years (Turjeman et al. 2021). This might be associated with the underlying medical conditions, such as diabetes (Risqiyah et al. 2022) or immune status of the patients along with some other risk factors including past infections and heavy antibiotic exposures, or previous medical procedures during hospitalization such as invasive device application (Kajihara et al. 2015). The profound changes in aging lead to the immunosenescence thereby increasing susceptibility to infections (Giefing-Kröll et al. 2015).

Patient'sgender is known as one of the risk factors in enterococcal infections. It was reported that male patients are more likely to have enterococcal infections than the female ones (Kajihara et al. 2015). However, the present study demonstrated enterococci had equal prevalence in male and female patients. Interestingly, *E. faecalis* isolates was more prevalently detected in male patients, whereas *E. faecium* was more predominant in female patients. In addition to social, behavioral, and other environmental factors, it is generally believed that genetic and hormonal factors have a role in the reported sex disparities in infectious diseases, including enterococcal bacteremia. In response to infectious agents, sex hormones in men regulate the balance between a sex-specific pro- or anti-inflammatory action less efficiently than in women, causing male immune responses to sepsis to be more severe and protracted, frequently producing systemic damage than in women (Correa-Martínez et al. 2021).

Based on the hospital wards, the distribution of enterococcal infections was predominantly in the internal medicine wards followed by surgical rooms. These results might indicate the prevalence of enterococci as the main causative agent responsible for healthcare associated infections. Furthermore, this may also indicate patients treated for particularly genitourinary or gastrointestinal abnormalities in the internal medicine wards in this study are more likely to have enterococcal infections. *E. faecalis* and *E. faecium* infections were associated with abnormal genitourinary anatomy and a gastrointestinal focus, respectively (Billington et al. 2014).

In conclusion, the occurrence of multidrug-resistant enterococci is prominent concerns leading to significant health and economic ramifications. The present study elucidated the distribution of two main enterococcal pathogens, namely *E. faecalis* and *E. faecium* in clinical samples. This study also evaluated the pattern of

enterococcal antibiotic susceptibility to determine the most effective antimicrobials as empirical therapy. Regular monitoring and surveillance along with improving and maintaining hygiene, strict adherence to antimicrobial stewardship is essential and should be effectively implemented to prevent enterococcal infection spread in communal and hospital settings.

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