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Original Article

Molecular Detection of Efflux Pump Genes and Antimicrobial Resistance Patterns among Uropathogenic Enterococcus Species

*Alam N[1], Nigar I[2], Ahmed I[3], Sultana A[4], Foyzunnahar M[5], Sarmin R[6], Ahmed S[7].

Abstract:

Background: Urinary tract infection (UTI) are among the most common infections caused by *Enterococcus* species, particularly *Enterococcus faecalis* and *Enterococcus faecium*. The clinical importance of enterococci is largely attributed to their intrinsic and acquired resistance to multiple antimicrobial agents. Efflux pump systems such as *emeA* and *efrAB* contribute significantly to multidrug resistant (MDR).

Methods: This cross-sectional laboratory-based study included 53 *Enterococcus* isolates obtained from urine samples of patients with clinically suspected UTI. Antimicrobial susceptibility testing was performed according to CLSI guidelines. MDR was defined as resistance to at least one agent in three or more antimicrobial classes. Detection of efflux pump genes (*emeA, efrA,* and *efrB*) was carried out using polymerase chain reaction (PCR).

Results: *E. faecalis* (81.1%) was the predominant species, followed by *E. faecium* (9.4%) among the isolates. High resistance rates were observed against tetracycline (94.3%), ciprofloxacin (66%), levofloxacin (62.3%), high-level gentamicin (54.7%), and benzyl penicillin (50.9%). Overall, 64.2% of isolates were identified as MDR. MDR was more frequent in *E. faecium* (80%) than *E. faecalis* (55.8%). The *emeA* gene was detected in 62.8% of *E. faecalis* and 100% of *E. faecium* isolates. All MDR isolates carried at least one efflux pump gene.

Conclusion: High prevalence of MDR *Enterococcus* species, particularly *E. faecium*, underscores the importance of efflux pumps in antimicrobial resistance. Rational antibiotic use and antimicrobial stewardship programs are essential to limit efflux-mediated resistance.

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Introduction:

Enterococci are normal commensal organisms which are generally considered to be low virulent in healthy individuals and have emerged as significant opportunistic pathogens, particularly in hospitalized patients.[1]

1. Dr. Nadia Alam, MBBS, MD (Microbiology), Lecturer, Department of Microbiology and Mycology, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka.
2. Dr. Ismet Nigar, Associate Professor, Department of Microbiology and Immunology, Bangladesh Medical University (BMU), Dhaka, Bangladesh.
3. Dr. Imran Ahmed, Assistant Professor, National Institute of Cardiovascular Disease (NICVD), Sher-E-Bangla Nagar, Dhaka, Bangladesh.
4. Dr. Abida Sultana, Assistant Professor, Department of Microbiology, City Medical College, Gazipur, Bangladesh.
5. Dr. Mst Foyzunnahar, Lecturer, Department of Occupational and Environmental Health, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka-1212, Bangladesh.
6. Dr. Rubayath Sarmin, Lecturer, Department of Maternal and Child Health, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka-1212, Bangladesh.
7. Dr. Sharmeen Ahmed, Professor (Retired), Department of Microbiology and Immunology, Bangladesh Medical University (BMU), Dhaka, Bangladesh.

Corresponding author: Dr. Nadia Alam,
MBBS, MD (Microbiology), Lecturer, Department of Microbiology and Mycology, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka
Email- dr.nadiarumpa@gmail.com.

The urinary tract is a common location for enterococcal infections.[2] Enterococci ranked third most common cause of hospital-acquired infections (HAI) after *Escherichia coli*, *Staphylococcus saprophyticus* and accounted for 11.56% of urinary tract infection in Bangladesh.[3] Another more recent study

reported that enterococci accounted for 6.4% among the urinary pathogens in which *E. coli* (51.6%) was the most prevalent isolates.[4]

E. faecalis and *E. faecium* are the most commonly identified *Enterococcus* species that are mostly responsible for human infection.[5] *E. faecalis* causes 80-90% of human enterococcal infections while *E. faecium* accounts for majority of the remainder.[1] Furthermore, less frequently isolated *Enterococcus* species like *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus* are usually found in the intestines of humans and animals, on the surfaces of plants, and in dairy products[6] which can carry transmittable antimicrobial resistance genes by horizontal gene transfer and contribute to the overall increase of antibiotic-resistant infections.[7]

Enterococci are important nosocomial pathogens that have both an intrinsic and acquired resistance to multiple antibiotics.[8] Increased resistance to amoxicillin (45.5%), ciprofloxacin (66%), nitrofurantoin (21.7%) and ceftriaxone (74.3%) was observed in the antibiotic resistance patterns of *Enterococcus* species in Bangladesh.[9] *E. faecium* exhibits a higher degree of resistance to all antimicrobial agents in comparison to *E. faecalis*.[10]

Multidrug resistant (MDR) bacteria are public health emergency that has a harmful impact on clinical outcomes. Multidrug resistance prolongs hospitalization time, increases treatment cost and increases the risk of treatment failure and death.[11] The *Enterococcus* isolate that is non-susceptible at least one agent in three or more antimicrobial classes (Aminoglycosides, Fluoroquinolone, Glycopeptides, Glycylcyclines, Lipopeptides, Oxazolidinones, Penicillin, Streptogramin) is known as MDR *Enterococcus*.[12] MDR *Enterococcus* species were found to be 35% in Bangladesh.[4]
As per Clinical and laboratory standard institute guideline, Fluoroquinolone such as ciprofloxacin and levofloxacin, and Nitrofurantoin (NIT) are specific for uropathogenic infection and have the potential to concentrate in urine.[13] However, the extensive usage of these drugs plays important role in development of MDR *Enterococcus* species.

The most extensively studied efflux pumps in *Enterococcus* species are *emeA,* homolog of *norA*, and *efrAB*, both belonging to the ATP binding cassette (ABC) superfamily of multidrug efflux transporters[14] and contribute to resistance to different antibiotics in *Enterococcus* species and to develop MDR.[15] In recent years, drug resistance in bacteria has received a lot of attention, particularly resistance to various antibacterial. Drug efflux, and particularly multidrug efflux, is emerging and contributing to the establishment of multidrug resistance in bacteria.[16]

In the perspective of previous discussion, the present study was designed to study the antimicrobial resistance profiles of different species for identification of MDR *Enterococcus* species along with detection of efflux pump genes among isolated *Enterococcus* species by PCR in Microbiology laboratory of Bangladesh Medical University (BMU).

Materials and method
This cross-sectional descriptive study was conducted from March 2023 to February 2024 in the Department of Microbiology and Immunology, Bangladesh Medical University (BMU), Dhaka. Ethical approval was obtained from the Institutional Review Board of BMU (Ref: BSMMU/2023/9044).

A total of 53 *Enterococcus* isolates obtained from overnight culture of urine samples having colony count $\geq 10^4$ CFU/ml[15] by purposive sampling and were used in this study by primary identification in blood agar and chromogenic agar media by their colony morphology and were confirmed by gram staining, catalase test, bile esculin test. Identification of species of enterococci were done by conventional biochemical tests such as fermentation of mannitol, sorbitol, raffinose, arabinose, utilization of pyruvate, arginine decarboxylase test.[17]

All the isolated *Enterococcus* species were subjected to antimicrobial susceptibility testing by both automated method (VITEK® 2 Compact) and Kirby-Bauer disc diffusion method.[18] Though VITEK® 2 Compact AST card P-628 couldn't report sensitivity of amoxicillin and Fosfomycin, so the antibiotic discs of amoxicillin (10 µg) and fosfomycin (200 µg) were used for disc diffusion tests (Biomaxima, Poland). The results were

interpreted as per recommendation of Clinical and Laboratory Standard Institute guideline.[13] Quality control (QC) strains used in this study was *E. faecalis* ATCC® 29212™.

The efflux pump genes were detected in the 53 enterococcal isolates by polymerase chain reaction (PCR). From pure cultures, 6 - 8 enterococcal colonies were randomly selected, diluted with 200 µL of double distilled water (ddH_2O), then boiled at 95°C for 10 min, followed by centrifugation at 12,000×g for 5 min, 100 µl supernatant of the solution contained the DNA of the enterococcal strains. The concentration of DNA was measured by spectrophotometric assay using a Nanodrop 2000 spectrophotometer (Thermo Fisher scientific, Waltham, MA, USA) according to manufacturer's instructions. The primers [15,19] were described previously and were purchased from Orbit Trade Company, Dhaka. PCR was performed with a 25 µl system containing 15 µl reaction mixture comprised of master mix with taq polymerase (Emerald Amp®MAX PCR master Mix) (Takara Bio, Japan), 5 µl DNA template, 1 µl of the forward and reverse primers, and 3 µl ddH_2O under the conditions mentioned in table 1.

Table 1: The PCR run protocol for efflux pump genes

Genes	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
emeA	94˚C/5 min	94˚C/45 sec	57˚C/60 sec	72˚C/90 sec	30	94˚C/5 min
efrA	94˚C/5 min	94˚C/45 sec	57˚C/45 sec	72˚C/45 sec	30	72˚C/5 min
efrB	94˚C/5 min	94˚C/45 sec	57˚C/45 sec	72˚C/45 sec	30	72˚C/5 min

The amplification products were electrophoresed on 1.5% agarose gels (SaeKem®Lanza, USA) and stained with 1% ethidium bromide (Applichem panreac, Spain) followed by electrophoresis at 120V for 48 minutes in horizontal electrophoresis apparatus (Biometra, Germany) containing ^{1}x TAE buffer and finally visualized by using a UV trans-illuminator (INFINITY-vilber Lourmat).

Descriptive analysis of all relevant variables was done by using frequency and percentage. Collected data were checked and analyzed with SPSS software package version-27 (Strata Corporation, College station, Texas).

Result

E. faecalis was the most frequent species (81.1%), followed by *E. faecium* (9.4%), *E. casseliflavus* (3.8%), *E. raffinosus* (3.8%), and *E. hirae* (1.9%) (Figure1).

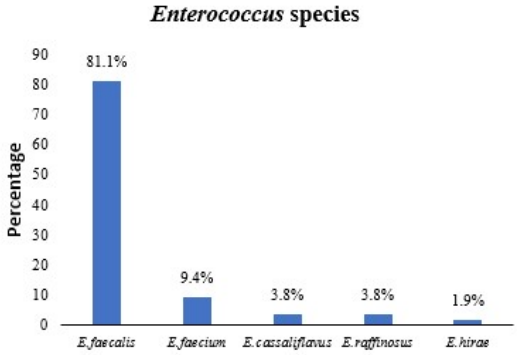

Figure 1: Distribution of the isolated uropathogenic *Enterococcus* species

No resistance to linezolid, vancomycin, teicoplanin and fosfomycin was detected among the 53 *Enterococcus* isolates, while over 50% prevalence of resistance to most antibiotics tested in this study was found. The majority of the isolated *Enterococcus* species exhibited resistance to tetracycline followed by ciprofloxacin, levofloxacin, high level gentamicin and penicillin (Table 2).

Table 2: Antimicrobial resistance pattern of isolated *Enterococcus* species (n = 53)

Antibiotics *	Number of resistant isolates n (%)					
	E.faecalis ** **(n = 43)**	***E.faecium*** **(n = 5)**	***E.casseliflavus*** **(n = 2)**	***E.raffinosus*** **(n = 2)**	***E.hirae*** **(n = 1)**	**Total (n = 53)**
Amoxicillin	14(32.6%)	3(60%)	2(100%)	1(50%)	1(100%)	21(39.6%)
Benzyl penicillin	20(46.5%)	3(60%)	2(100%)	1(50%)	1(100%)	27(50.9%)
High level gentamicin	22(51.2%)	4(80%)	2(100%)	0(0%)	1(100%)	29(54.7%)
Ciprofloxacin	**27(62.8%)**	**4(80%)**	**2(100%)**	**2(100%)**	**0(0%)**	**35(66%)**
Levofloxacin	**25(58.1%)**	**4(80%)**	**2(100%)**	**2(100%)**	**0(0%)**	**33(62.3%)**
Nitrofurantoin	**04(9.3%)**	**1(20%)**	**2(100%)**	**0(0%)**	**0(0%)**	**7(13.2%)**
Vancomycin	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Teicoplanin	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Tetracycline	40(93.0%)	5(100%)	2(100%)	2(100%)	1(100%)	50(94.3%)
Linezolid	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Fosfomycin	0(0%)	NT	NT	NT	NT	0(0%)

*Antibiogram selected according to CLSI 2023
**Fosfomycin susceptibility was observed only among *E. faecalis* isolates as per CLSI, 2023.
***Not tested (NT)

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MDR was observed in 64.2% of isolates, with higher prevalence in *E. faecium* (80%) than *E. faecalis* (55.8%) among 53 isolated *Enterococcus* species. Uncommon enterococcal species also exhibited higher number of MDR (Figure 2).

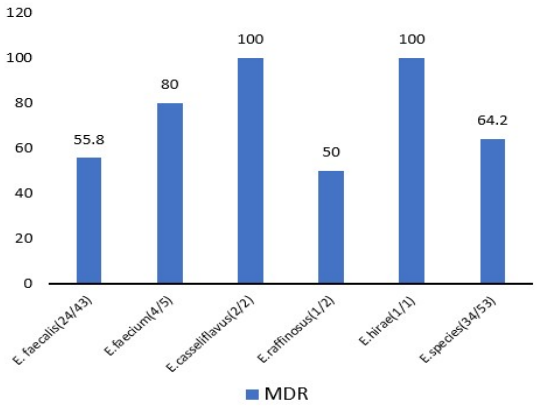

Figure 2: Distribution of MDR among uropathogenic *Enterococcus* species

Among total 34 isolated MDR, 91.4% isolates exhibited ciprofloxacin resistance, 88.6% showed levofloxacin resistance and only 20.6% exhibited nitrofurantoin resistant. The enterococcal isolates that were identified in low frequencies, among them the rate of producing of MDR was found to be high. Fluoroquinolones resistance mostly contributed to the development of MDR in *Enterococcus* species. Uncommon enterococcal species were few in urinary isolates, but they showed more MDR than the large number of isolated *E. faecalis* (Figure 3 & 4).

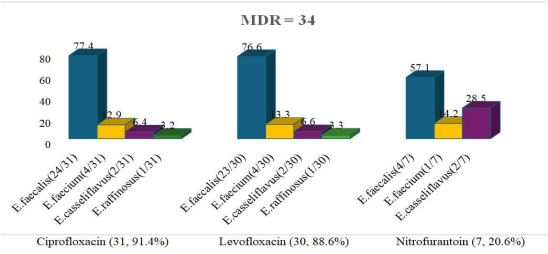

Figure 3: Distribution of fluoroquinolone and nitrofurantoin resistant *Enterococcus* species among MDR isolates

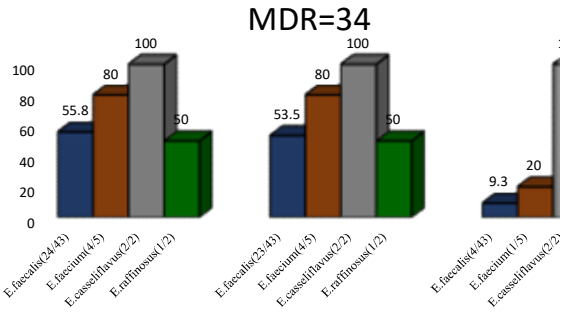

Figure 4: Rate of MDR among different species of *Enterococcus*

Among 43 isolated *E. faecalis, emeA*, *efrA* and *efrB* genes were found in 27(62.8%), 26(60.5%) and 15(34.9%) isolates respectively. All 5 isolates of *E. faecium* (100%) had *emeA* and *efrA* genes. But *efrB* gene was found in 4(80%) isolated *E.faecium*. Among the isolated *E. casseliflavus*, *E. raffinosus* and *E. hirae*, efflux pump genes were not detected.
All the MDR enterococcal isolates harbored either the efflux pump gene, *emeA* or *efrAB*. Most of the *E. faecium* isolates harbored MDR efflux pump genes (Table 3).

Table 2: Presence of MDR efflux pump genes among isolated *Enterococcus* by PCR (n = 48)

MDR efflux pump genes	*E. faecalis* (43) n (%)	*E. faecium* (5) n (%)
emeA	27(62.8%)	05(100%)
efrA	26(60.5%)	05(100%)
efrB	15(34.9%)	04(80%)

Discussion:
Urinary tract infection (UTI) constitutes the most common type of clinical disease, caused by enterococci, both within and outside hospital settings.[20] The clinical importance of the genus *Enterococcus* is directly related to its antibiotic resistance.[21]

The present study was designed to detect the MDR efflux pump genes (*emeA*, *efrA*, *efrB*) by PCR along with evaluation of enterococcal antibiotic resistance profile to identify the MDR *Enterococcus* species. Special attention was given on the fluoroquinolones & nitrofurantoin resistance, as these antibiotics are convenient therapeutic agents that are advised for the treatment of enterococcal UTI and contribute to develop MDR.[2]

In this study, out of 53 isolated *Enterococcus* species, *E. faecalis* (81.1%) was found to be the most common species followed by *E. faecium* (9.4%), *E. casseliflavus* (3.8%), *E. raffinosus* (3.8%) and *E. hirae* (1.9%). Another study stated that *E. faecalis* and *E. faecium* mostly responsible for human infection.[5] In Bangladesh, a previous study demonstrated that *E.faecalis* was 71.18% in urine specimen followed by *E.faecium* which accounted for 16.94%, *E.avium* was 2.43%, *E.raffinosus* 2.43%.[1] The discrepancy may be due to changes in hospital environmental factors such as air movement, humidity, temperature and cleanliness significantly contributing to bacterial concentration.[22]

Enterococci exhibit intrinsic and acquired resistance to various antibiotics, such as β-lactams, aminoglycosides, macrolides, lincosamides, cotrimoxazole, glycopeptides.[20] Knowledge of the antimicrobial resistance profile is essential to formulate treatment guidelines for infections caused by *Enterococcus* species.

In this study, irrespective of species, most of the *Enterococcus* isolates exhibited increased resistance to ciprofloxacin (66%), levofloxacin (62.3%), tetracycline (94.5%), high level gentamicin (54.7%), benzyl penicillin (50.9%) and amoxicillin (39.6%). These findings are near to the study in Iran which also exhibited increased resistance of *Enterococcus* species to ciprofloxacin (65.4%), high level gentamicin (60.1%), benzyl penicillin (68.6%) and ampicillin (28.2%).[23]

In the current study, total ciprofloxacin and levofloxacin resistant enterococcal isolates were found to be 66% and 62.3%, respectively. A study in Bangladesh observed 66% ciprofloxacin resistant *Enterococcus* isolates that is similar to this study, but levofloxacin resistance was not reported by them.[9] Higher resistance rate to fluoroquinolone may be due to use of fluoroquinolones as empirical therapy for UTI.[24]

The present study showed, 13.4% enterococcal isolates were resistant to nitrofurantoin. Study from Bangladesh, showed similar report in which 15.3% enterococcal isolates were resistant to nitrofurantoin. The extensive use of nitrofurantoin in UTI, has resulted in the increased drug resistance of *Enterococcus* species.[19]

According to CLSI,2023, tetracycline is used only primarily in urinary tract infection in case of *Enterococcus*.[13] A Study in Saudi Arabia, reported 86.36% *E. faecalis* to be tetracycline resistant which showed similar findings to that of the present study.[25] The use of antibiotic in food and animal production was mostly responsible for antibiotic resistance among WHO South-East Asia Region. They stated that in Bangladesh, the mostly used antibiotics in aquaculture, farming and poultry were tetracyclines.[26] This may be the cause of increased resistance to tetracycline in Bangladesh.

In this present study, *E. faecium* isolates showed more resistance to the commonly used antimicrobial agents in comparison to *E. faecalis*. It is also demonstrated that *E. faecium* strains displayed a higher degree of resistance to all antibiotics as compared to *E. faecalis.*[10, 23]

In the current study, among 53 isolated *Enterococcus* species, 64.2% were found to be MDR. In Iran, research reported 79.3% of MDR enterococcal isolates causing UTI.[23] Another study also found 66.6% of MDR in nosocomial enterococcal isolates from all clinical specimens. The findings are nearly similar to this study.[27]

In the present study, MDR *E. faecalis* were 55.8% and *E. faecium* were 80%. A study from Egypt reported 74.6% *E. faecalis* and 100% of *E. faecium* isolates were MDR which is little bit higher than this study.[28] This difference may occur due to geographical variation that has role in antimicrobial susceptibility and resistance patterns of enterococci species.[29] Lack of standard protocols, empirical use of antibiotics, and lack of sufficient information are the leading causes for the emergence and spread of MDR enterococci in hospitals.[28]

In this study, less commonly isolated *Enterococcus* species such as *E. casseliflavus*, *E. raffinosus* and *E. hirae* in urine showed higher percentages of MDR in comparison to the large number of isolated *E. faecalis*. Less

frequently isolated *Enterococcus* species are found in meat products and animal feces, carrying transmittable antimicrobial resistance genes.[7, 30]

In India, a study presented that all of identified *E. casseliflavus* isolates (100%) from urine, was found to be MDR.[10] Again, in Algeria, research reported on *E. hirae* in different clinical specimens including urine which showed that this rarely isolated organism was found to be MDR. Both of which is completely similar to this study.[30]

Among total 34 isolated MDR, 91.4% and 88.5% isolates exhibited ciprofloxacin and levofloxacin resistance respectively. In India, a study revealed that 90.08% isolates exhibited ciprofloxacin resistance among MDR *Enterococcus* isolates.[31] In the present study, all the 7 nitrofurantoin resistant isolates were found to be MDR. Nitrofurantoin resistance in *Enterococcus* species is relatively less in comparison to other first line antimicrobial agents but, it contributes much to produce MDR. In Bangladesh poor healthcare standards, along with the misuse and overuse of antibiotics contributes to increasing MDR.[9]

In the current study, *emeA* gene was harbored in 62.8% *E. faecalis* and 100% *E. faecium* isolates. A study of Iraq presented that all *E. faecalis* isolates exhibited MDR and *emeA* was distributed (95%) among *E. faecalis* isolates.[32] While other studies reported that the gene *emeA* was present in all uropathogenic isolates.[33] Another study indicated that prevalence of *efrA* and *efrB* genes in all of isolates of *E. faecalis* is 100%. MDR efflux pump gene *efrAB* is a heterodimeric ABC transporter, which can cause drug resistance when *efrA* and *efrB* genes are expressed together and it results increasing drug resistance.[15] In this study, all the MDR enterococcal isolates harbored either one of the MDR efflux pump genes, *emeA* or *efrAB* and most of the *E. faecium* isolates harbored MDR efflux pump genes. *Enterococcus* species shows high levels of resistance to many antimicrobial agents, presumably due to the presence of efflux pumps.[34]

The study finding proves that the presence of efflux pump contributes to the development of MDR. The selection of efflux pump overproducing strains is influenced by bacterial exposure to antibacterial, and limiting such exposure, including minimizing the antibacterial usage, would limit the occurrence of efflux-mediated drug resistance.

Conclusion:

This study demonstrates a considerable prevalence of MDR *Enterococcus* species among urine samples, with *Enterococcus faecalis* being the most common species and *Enterococcus faecium* exhibiting comparatively higher resistance rates. High levels of resistance were observed against fluoroquinolones, tetracycline, and aminoglycosides, while nitrofurantoin resistance, though lower, was exclusively associated with MDR isolates. Importantly, the detection of efflux pump genes (*emeA* and *efrAB*) in all MDR isolates confirms their significant role in mediating antimicrobial resistance. These findings highlight the urgent need for rational antibiotic prescribing, strengthened antimicrobial stewardship programs, and continuous resistance surveillance to limit the emergence and spread of MDR enterococci. Hospitals should restrict fluoroquinolone use for uncomplicated UTI given > 60% resistance and prioritize nitrofurantoin and fosfomycin based on local susceptibility.

Limitation

The study was conducted at a single center, which may limit the generalizability of the findings. In addition, the relatively small sample size may have reduced the statistical power of the study.

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Ethical statement:

This study was reviewed and approved by the Institutional Review Board of BMU. The informed consent form was exempted by the IRB, as the study samples were laboratory isolates.

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Conflict of Interest

The authors report no conflicts of interest in this work.

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