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RESEARCH PAPER

Deciphering the Nitrofurantoin Resistance Mechanism in Uropathogenic *Enterococcus* Species

Nadia Alam[1], Ismet Nigar[2], Imran Ahmed[3], Abida Sultana[4], Sharmeen Ahmed[2]

[1]Department of Microbiology and Mycology, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka, [2]Department of Microbiology and Immunology, Bangladesh Medical University (BMU), Dhaka, [3]National Institute of Cardiovascular Disease (NICVD), Dhaka, [4]Department of Microbiology, City Medical College, Gazipur,

Abstract

Background: Urinary tract infection caused by *Enterococcus* species is of great concern both in community and hospital settings due to their increasing resistance to multiple antimicrobial agents. Nitrofurantoin (NIT) is an important therapeutic option for urinary tract infection caused by *Enterococcus* species, whose mechanism of resistance remains to be explored.

Objectives: To evaluate status of nitrofurantoin resistance in *Enterococcus* species by detection of efflux pump genes (*emeA*, *efrA*, *efrB*) and nitroreductase genes (*ef0404*, *ef0648*) and assessing the effect of efflux pump inhibitors (verapamil and chlorpromazine) on reducing the minimum inhibitory concentrations (MICs) of nitrofurantoin in resistant isolates.

Methods: Laboratory isolated *Enterococcus* species were identified from culture of urine specimen in Department of Microbiology and Immunology, Bangladesh Medical University (BMU). These isolates were subjected to nitrofurantoin susceptibility tests by both broth microdilution technique and automated method (VITEK®2 Compact). Enterococcal efflux pump genes (*emeA*, *efrA* and *efrB*) and nitroreductase genes (*ef0404*, *ef0648*) were detected by conventional PCR. Finally, the MICs of nitrofurantoin with and without efflux pump inhibitors (verapamil and chlorpromazine) were determined in resistant isolates by broth microdilution method.

Results: Among the 53 *Enterococcus* species isolated from urine, majority were *E. faecalis* (81.1%). Nitrofurantoin resistant isolates were 13.2% and mostly exhibited by the rarer species. At least one of the nitroreductase genes was present in all of the nitrofurantoin-susceptible enterococci isolates (*ef0404*, 80.4% and *ef0648*, 91.3%). Additionally, *emeA* and *efrAB* genes were present in 65.2% and 34.8% of the nitrofurantoin-susceptible isolates, respectively. The isolates resistant to nitrofurantoin did not contain any nitroreductase genes. Efflux pump genes were present in less than half (40%) of the resistant isolates. Effective reduction of MIC of nitrofurantoin among nitrofurantoin resistant enterococci isolates were observed in the combination of nitrofurantoin and chlorpromazine in comparison to nitrofurantoin and verapamil combination.

Conclusion: Deletion in the nitroreductases-encoding genes was the main reason for nitrofurantoin resistance in *Enterococcus* species rather than presence of efflux gene. The reduction of MIC of nitrofurantoin by chlorpromazine a efflux pump inhibitor advocate adjuvant role of it to overcome nitrofurantoin resistance.

Key words: *Enterococcus*, efflux pump genes, nitroreductases genes, efflux pump inhibitor, nitrofurantoin, chlorpromazine.

Introduction

Enterococci have recently been identified as one of the leading causes of highly challenging health care associated infections due to their extensive drug resistance. A common location for enterococcal infections is the urinary tract.[1] In Bangladesh, uropathogenic enterococci accounted for 6.4% infection.[2] The most frequently identified *Enterococcus* species, *E. faecalis* and *E. faecium*, are mainly responsible for the development of human infections.[3] *E. faecalis* causes 80-90% of human enterococcal infections while *E. faecium* accounts for majority of the remainder.[4] Rarer *Enterococcus* species have been associated with infections of immunocompromised individuals and can spread antibiotic-resistant genes through horizontal gene transfer.[5]

***Correspondence:** Nadia Alam, Department of Microbiology and Mycology, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka-1212*

e-mail: dr.nadiarumpa@gmail.com

ORCID ID: 0009-0008-7554-6725

The Clinical and Laboratory Standards Institute (CLSI) guideline 2023, describes that nitrofurantoin (NIT) is specifically used for uropathogenic organisms. Microbial nitroreductase enzymes reduce the nitro group of NIT to form a cytotoxic nitro radical. This highly reactive nitro radical interacts with bacterial DNA and causes structural damage. The resulting DNA damage leads to bacterial cell death and produces the antibacterial effect. The primary causes of NIT resistance in *Enterococcus* species are deletions in the nitroreductases-encoding genes *ef0404* and *ef0648* and the overexpression of efflux pump genes.[13]

Efflux pumps reduce the accumulation of antibiotics inside of the bacteria.[6] The most studied efflux pumps in *Enterococcus* species are *emeA*, belongs to the major facilitator superfamily (MFS), and *efrAB*, member of the ATP-binding cassette (ABC) superfamily of multidrug efflux transporters.[7] Efflux pump inhibitors (EPIs) are the molecules cause inactivation of drug transport by inhibiting bacterial efflux pumps.[8] Necessary characteristics of a pump inhibitor are that it can be active at an attainable serum drug concentration and that it has minimal toxicity.[9] These compounds can be used in combination with the relevant antibiotics to achieve synergistic action.[10]

However, the extensive usage of NIT has resulted in rise of drug resistance.[11] In Bangladesh, resistance to NIT was found in 10.5% of enterococci.[12] Verapamil (100 µg/ml) and chlorpromazine (20 µg/ml) are known efflux pump inhibitors for *Enterococcus* species[13] which are available in Bangladesh for treatment of hypertension and schizophrenia respectively.

In the perspective of previous discussion, our study was intended to identify the efflux pump genes and nitrofurantoin resistant related genes to identify nitrofurantoin resistance mechanism. Study of nitrofurantoin resistance mechanisms will be also helpful in establishing a theoretical basis for the rational use of this drug and in adopting appropriate control measures to decrease resistance. Our aim was also to evaluate the effects of EPIs (verapamil & chlorpromazine) on nitrofurantoin resistant isolated uropathogenic *Enterococcus* species. The reversal of this efflux mediated resistance, via inhibition of drug efflux mechanisms, is a promising research area. The reason for developing EPIs is the urgent need to discover small molecules, which can be combined with conventional antibiotics to block multidrug efflux systems.

Materials and Method

The cross-sectional descriptive study was conducted from March 2023 to February 2024 in the laboratory of the Department of Microbiology and Immunology, Bangladesh Medical University (BMU), Dhaka.

Sampling technique was purposive sampling and ethical consideration was taken from Institutional Review Board of BMU. Total 53 *Enterococcus* species isolated from overnight culture of urine samples having colony count $\geq 10^4$ CFU/ml[15] 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.[16]

All isolated *Enterococcus* species were subjected to nitrofurantoin susceptibility testing by both broth microdilution technique and automated method (VITEK® 2 Compact) with AST P628 (Ref. no 414534) cards and the results were interpreted according to the recommendations of the CLSI, 2023 guidelines. Quality control (QC) strains used in this study was *E. faecalis* ATCC® 29212™.

The efflux pump genes and nitrofurantoin resistance related 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 ddH_2O, then boiled at 95°C for 10 min, followed by centrifugation at 12,000 r/min 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[7,13,17] 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 ddH2O under the conditions mentioned in table I.

Table I: The PCR run protocol for efflux pump genes and nitrofurantoin resistance related genes

Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
Efflux pump genes (*emeA*, *efrA* and *efrB*)					
94°C/5 min	94°C/45 sec	57°C/60 sec	72°C/90 sec	30	94°C/5 min
Nitrofurantoin resistance related genes (*ef0404* and *ef0648)*					
98°C/30 sec	98°C/10 sec	59°C/30 sec	72°C/30 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).

MICs of nitrofurantoin (Incepta Pharmaceuticals Ltd) were determined by broth microdilution technique according CLSI M07-A9 Vol. 32 guidelines (ISO 20776-1:2006). In two-fold broth dilution method, cation adjusted Mueller Hinton broth (Himedia, India), with an inoculum size of approximately 5×10^{5} CFU/ml, were supplemented with serial concentrations of nitrofurantoin (4-512 µg/ml) with and without the either efflux pump inhibitors verapamil (Incepta Pharmaceuticals Ltd; 100 µg/ml), and chlorpromazine (Opsonin Pharma Limited; 20 µg/ml), dissolved in distilled water.[18] Plates were incubated at 37°C and the MICs were recorded after 24 hours as the lowest concentration of test compound that was able to inhibit the visual growth.

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).

Results

Enterococcus faecalis (81.1%) was the most frequently isolated enterococci followed by *Enterococcus faecium* (9.4%; Figure1). Among all enterococcal isolates, 13.2% were found to be resistant to nitrofurantoin (Figure 2). Nitrofurantoin resistance was observed in 9.3% of *E. faecalis* isolates, 20% of *E. faecium* isolates, and 100% of *E. casseliflavus* isolates (Figure 3).

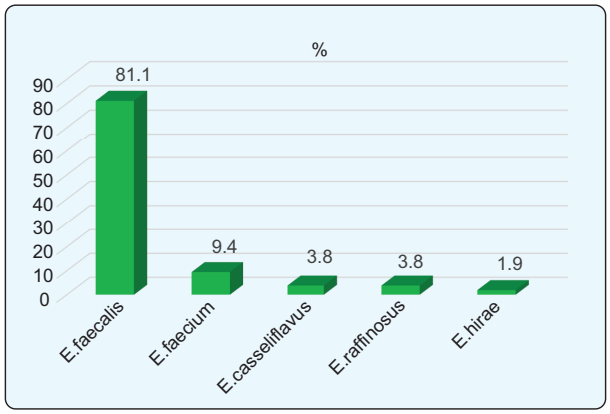

Figure 1: Distribution of the isolated uropathogenic *Enterococcus* species (N=53)

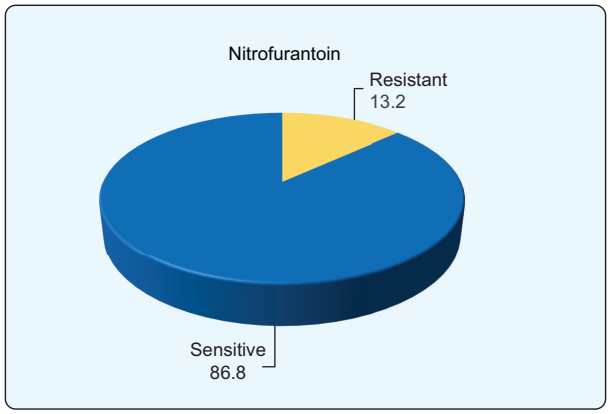

Figure 2: Nitrofurantoin resistance of *Enterococcus* species (N=53)

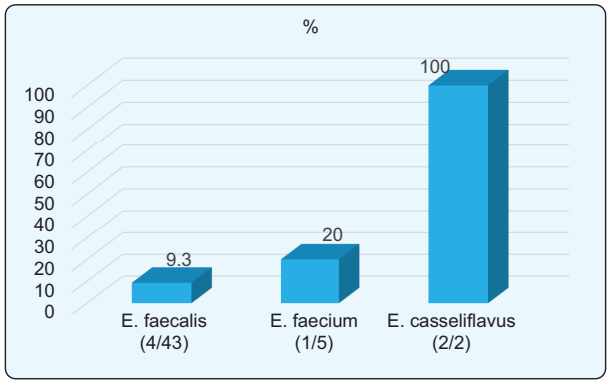

Figure 3: Rate of Nitrofurantoin resistance among different species of *Enterococcus* (N=7)

All the nitrofurantoin susceptible enterococci isolates were found to harbor at least one of the nitroreductase genes (*ef0404*-80.4% and *ef0648*-91.3%), and none of the nitroreductase genes were detected in the nitrofurantoin (NIT) intermediate and resistant isolates.

Furthermore, this study revealed that among all nitrofurantoin susceptible *enterococcal* isolates, efflux pump gene, *emeA* and *efrAB* were carried by 65.2% and 34.8% isolates respectively (Table II). Table III denotes that, highest MIC value for nitrofurantoin in nitrofurantoin resistant isolates were found to be 256 µg/ml, which were observed in 3.8% isolates (Nitrofurantoin: Sensitive ≤32 µg/ml, Intermediate 64 µg/ml, Resistant ≥128 µg/ml) .

Effective reduction of MIC of nitrofurantoin among nitrofurantoin resistant enterococci isolates was observed in the combination of nitrofurantoin and chlorpromazine in comparison to nitrofurantoin and verapamil combination (Table IV).

Table II: Distribution of NIT resistance-related genes and efflux pump genes in Nitrofurantoin Susceptible (NIT-S), Intermediate (NIT-I) and Resistant (NIT-R) *Enterococcus* isolates by PCR (N=50)

NIT response in	Nitroreductase genes		Efflux pump genes	
Enterococcus isolates	*ef0404* gene%	*ef0648* gene%	*emeA* %	*efrAB*%
NIT- Susceptible (n=46)	80.4%	91.3%	65.2%	34.8%
NIT- Intermediate (n=2)	0%	0%	50%	50%
NIT- Resistant (n=2)	0%	0%	20%	40%

Table III: Minimum inhibitory concentration (MIC) of nitrofurantoin by broth microdilution among *Enterococcus* isolates (N=53)

MIC values of nitrofurantoin (µg/ml)	Number of *Enterococcus* isolates n (%)
8	11(20.8%)
16	20(37.8%)
32	15(28.3%)
64	02(3.8%)
128	03(1.9%)
256	02(3.8%)
512	0(0%)
Total	53(100%)

Nitrofurantoin: Sensitive ≤32 µg/ml, Intermediate 64 µg/ml, Resistant ≥128 µg/ml

Table IV. Minimum Inhibitory Concentrations (MICs) of Nitrofurantoin in NIT -Resistant and NIT-Intermediate isolates with or without the efflux pump inhibitors (N=4)

Enterococcus Isolates	Nitrofurantoin MICs (µg/ml)		
	Nitrofurantoin	NIT + Verapamil (100µg/mL)	NIT + Chlorpromazine (20µg/mL)
Resistant isolates (N = 2)			
Isolate 1	256	128	128
Isolate 2	256	128	64
Intermediate isolates (n = 2)			
Isolate 1	64	32	64
Isolate 2	64	32	32

Discussion

Urinary tract infection (UTI) constitutes the most common type of clinical disease, caused by enterococci, both within and outside hospital settings.[19] Nitrofurantoin are convenient therapeutic agents that are advised for the treatment of enterococcal UTI.[1]

We investigated the main mechanism of NIT resistance in *Enterococcus* species, which is mediated by nitroreductase genes and multidrug resistance efflux pump gene. Along with that, we evaluated effects of efflux pump inhibitors (verapamil & chlorpromazine) on the nitrofurantoin resistant uropathogenic enterococcal isolates.

The present study showed that 13.2% enterococcal isolates were resistant to nitrofurantoin. Another Study from Bangladesh, showed a similar report in which 15.3% enterococcal isolates were resistant to nitrofurantoin.[4] Our finding was that, 5 nitrofurantoin resistant isolates had MIC value for nitrofurantoin within 128 µg/ml to 256 µg/ml. Similar finding was also observed.[13] Bacterial nitroreductases are flavoenzymes capable of reducing nitro compounds. Antimicrobial effect of nitrofurantoin is mediated by microbial nitroreductases that reduce the drug to a cytotoxic nitro radical and result in DNA damage.[11]

In our study, all the nitrofurantoin susceptible enterococcal isolates were found to harbor at least one of the nitroreductase genes (*ef0404*-80.4% and *ef0648*-91.3%), and none of the nitroreductase genes were found among the nitrofurantoin intermediate and resistant isolates. Previous study[13] revealed that, all the susceptible isolates were found to carry at least one nitroreductase gene (*ef0404* 75.0% and *ef0648* 72.5%) which is similar to our study. Though nitroreductase genes were not detected in nitrofurantoin intermediate and resistant isolates by us, the carriage rates of the nitroreductase genes among the NIT-intermediate and resistant isolates were 50% and 20%, respectively.[13] The discrepancy may be due to the large number of samples in their study.

Again, among nitrofurantoin susceptible enterococcal isolates, 65.2% isolates carried *emeA* and 34.8% isolates carried *efrAB* gene. Carriage rate of *emeA* and *efrAB* genes among nitrofurantoin resistant isolates were 20% and 40%, respectively. Between the two resistant isolates, one isolate harbored only *efrAB* gene and another isolate harbored both *emeA* and *efrAB* genes.

Furthermore, the previous study described that, both nitrofurantoin sensitive and resistant *E. faecium* harbored efflux pump genes, *emeA* and *efrAB*, in 12.5% isolates.[13] Higher number of efflux pump genes in nitrofurantoin susceptible isolates may occur because of the inclusion of both *E. faecalis* and *E. faecium* in our study. These higher number of efflux pump genes in susceptible isolates in current study signifies that, the role of efflux pump genes in nitrofurantoin resistance is less important, rather than nitroreductase gene deletion play the major role.

Among nitrofurantoin resistant enterococci isolates, effective reduction that is four-fold reduction of MIC of nitrofurantoin were found when nitrofurantoin was supplemented with chlorpromazine in comparison to nitrofurantoin and verapamil combination. Previously the study interpretated that, 40% nitrofurantoin resistant isolates demonstrated a four-fold decrease in the MIC of nitrofurantoin in the presence of the efflux pump inhibitors.[13] This dissimilarity may be due to their very large sample size and inclusion of only *E. faecium*.

We can interpret that the efflux pumps genes *emeA* and *efrAB* do not play a significant role in nitrofurantoin resistance in the isolated species. Rather than deletions in the nitroreductase-encoding genes (*ef0404* and *ef0648*) are the main reasons for nitrofurantoin resistance in *Enterococcus* species.

The limitation of our study includes, inability to differentiate between community and hospital acquired enterococcal infection and evaluation of few important nitroreductase genes and efflux pump genes due to budget and time constraint.

Conclusion

E. faecalis was the most prevalent species among the identified uropathogenic enterococci. The study may pose a hypothesis that deletions in the nitroreductases-encoding genes *ef0404* and *ef0648* in nitrofurantoin resistant enterococcal isolates are mainly responsible for nitrofurantoin resistance in *Enterococcus* species. Though the efflux pump does not play significant role in nitrofurantoin resistant, effective reduction of MIC of nitrofurantoin was found when nitrofurantoin was supplemented with chlorpromazine in comparison to nitrofurantoin and verapamil combination as efflux pump inhibitor. Our findings would be useful in developing a theoretical framework for sensible use of NIT and in implementing suitable controls to stop the rise in antibiotic resistance.

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