

# Prevalence of Fosfomycin Resistant *Enterococcus faecalis* and detection of FosB gene in Tertiary Care Hospital, Chhattisgarh, India

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## Abstract:

**Introduction:** Urinary Tract Infection is the commonest infection in the community acquired and as well as hospital set up. Mechanisms of antimicrobial resistance in *Enterococcus* species are a significant concern. **Objective:** The aims and objective of our study was to know the prevalence of *Enterococcus* species as an uropathogens and fosfomycin resistance and the prevalence of FosB gene by sequencing in *Enterococcus faecalis*. **Material and Method:** We modified a PPF disk (Fosfomycin with sodium phosphonoformate) based on disk potentiation test which is expanded the inhibition zone around the PPF disc in the presence of FRGST producers. The production of (FR-GSTs) in the FREf strain using PPF disc was negative for FR-GSTs. Further fosfomycin resistant *Enterococcus faecalis* was confirmed by using PCR. Identified FREf were harbour with fosB gene. **Result:** In this study, Out of 618, 49(7.93%) specimens were significant with *Enterococcus faecalis* clinical isolates, 9 *Enterococcus faecalis* were resistant to fosfomycin (MIC  $\geq$ 512  $\mu$ g/l), 4 strains were intermediates to fosfomycin (MIC=128  $\mu$ g/l) and 36 were susceptible to fosfomycin (MIC  $\leq$ 64). The prevalence of 18.4% fosfomycin resistant *Enterococcus faecalis* (FREf) was found in our study. **Conclusion:** This finding prove that high expression level of fosB gene and Modification in the fosfomycin target enzyme MurA were the main reason of resistant mechanisms in these fosfomycin resistant *Enterococcus faecalis*. There are many studies done on the fosfomycin drug susceptibility and resistance mechanisms but still not completed. So, further, must urgently more research on fosfomycin resistant mechanism FosB gene

**Keywords:** Fosfomycin resistant, FosB gene, Disc potentiation test

**Introduction:**

Urinary Tract Infection is the commonest infection in the community acquired and as well as hospital set up. UTI prolonged the hospital stay, increase the morbidity and financial burden. *Enterococcus species* is the one of the leading uropathogen. It contributes 5% community - acquired UTI and 30% catheter associated UTI are caused by *Enterococcus spp.* <sup>(1)</sup>. Emergence of antibiotic resistance *Enterococcus species* are a significant concern, mainly highly effective high level gentamicin and vancomycin antibiotics. Resistant *Enterococcus species* against to high level gentamicin and vancomycin make fosfomycin one of the potential alternative antibiotic for treatment of the UTI <sup>(2)</sup>. Fosfomycin is bactericidal in nature that interferes in cell wall synthesis by inhibiting phosphoenolpyruvate synthesis <sup>(3)</sup>. It acts against both gram positive and gram negative organisms <sup>(4)</sup>. Now emergence of drug modifying enzymes, mutation of target enzyme MurA and *mutation* in chromosomal gene responsible for fosfomycin transporter results in emergence of fosfomycin resistance. Fos A, FosB, FosX and FosC are 4 types of fosfomycin modifying enzymes are present which catalyse the development of glutathionine fosfomycin ( FosA), L-cystein, fosfomycin ( FosB), H<sub>2</sub>O fosfomycin (FosX) and ATP fosfomycin (FosC). In gram positive organisms like *Enterococcus species* FosB is implicated which is a divalent thiol-s-Transferase. FosB are of different types FosB1-3, which are both chromosomal and plasmid mediated <sup>(5, 6)</sup> Fosfomycin is an approved drug only for treating UTI mainly *Escherichia coli* and *Enterococcus faecalis* <sup>(7)</sup>. Its resistance has been reported from different part of world like China but in India it is seldom reported.

The aims and objective of our study was to know the prevalence of *Enterococcus species* as an urpathogens and fosfomycin resistance and the prevalence of FosB gene by sequencing in *Enterococcus faecalis*.

**Material and Method:**

Urine specimen was collected from the patient of various wards and departments of Chhattisgarh Medical Science Hospital, Bilaspur, Chhattisgarh, India. This study was conducted at the period of, September 2018 to February 2019. A total 618 specimen were processed and identified, 49 (7.93%) specimens were significant with *Enterococcus faecalis*. Isolation and identification of organisms were done by as per the standard operating procedure <sup>(8)</sup>. The MIC of fosfomycin was done onto each isolated (N=49) *Enterococcus faecalis*. Results were interpreted according to CLSI guidelines <sup>(7)</sup>. Each Fosfomycin Resistance *Enterococcus faecalis* (FREf) strains were further tested by disc potentiation method by using sodium phosphonoformate disc (PPF) and Molecular study was done of fosfomycin resistant strains by PCR and nucleotide sequencing using specific primers.

**Susceptibility testing:** Each *Enterococcus faecalis* (N=49) were inoculated on to MHG6P plate (Mueller Hinton agar nwith supplemented Glucosae-6-Phosphate (25µg/ml) (MHG6P) (HiMedia) and Fosfomycin MIC™ strip (HiMedia) were placed and incubated for 24 hours at 37°C temperature. Results of MIC were interpreted according CLSI as shown in **Table no 2**. For the fosfomycin determination *Enterococcus faecalis* ATCC 29212 was applied *Enterococcus faecalis* ATCC 29212 was used as a quality control for the fosfomycin determination.

**Disc Potentiation Test:** Fosfomycin Resistant *Enterococcus faecalis* (*FREf*) were inoculated onto a MHG6P agar plate. The addition of glucose-6-phosphate to the Mueller Hinton agar is done according CLSI guidelines <sup>(7)</sup>. Two Kirby Bauer discs containing fosfomycin (50µg/disc) and G6P (5µg/disc) (Himedia) were applied on the agar plates. Sodium phosphonoformate Sodium Phosphonoformate (Aldrich) was dissolved in distilled water to a concentration of 50mg/ml and was applied (20 µl, 1 mg in total) in one of two fosfomycin discs and incubated for 18 hours at 37°C temperature and after incubation measured the increased diameter of inhibition zone around the PPF disc and compared with fosfomycin disc.

**Sample processing and DNA Extraction:** To isolate the DNA of microbial community, the bacterial samples were cultured in nutrient broth for overnight in incubator shaker at 37°C and 120rpm. The bacterial cells were pellet down using high speed centrifuge (Remi). The resulting pellet was used for total DNA extraction by the QIAprep Spin Miniprep Kit (cat No: 27104). Further, DNA samples were checked on agarose gel and spectrophotometer to analyze the quality and quantity of the extracted DNA <sup>(9)</sup>. The obtained sequence was analyzed in bioinformatics tools like BLAST on NCBI website and European Nucleotide Archive.

**PCR amplification:** To confirmation of *Enterococcus faecalis* strain contained each samples, the PCR was performed to amplify the 16S rDNA using the specific primers as shown table 1. Gel- electrophoresis was used to check the presence of a single band in an expected in size 1496 bps for every PCR product. The PCR products were purified by the pureLink PCR purification kit (Invitrogen, Lithuania) <sup>(10,11)</sup>. Further, plasmid was obtained culture and PCR against the fosB gene was performed <sup>(2)</sup>. Multiple bands are obtained and a band of 331 bps cut down from gel and eluted the PCR product for sequencing. Further, eluted product was used in sequencing. PCR was performed to amplify fosB using the specific primers as shown table 1.

Obtained sequence used in blast and which is showing homology with plasmid belongs to *Enterococcus faecalis*..

### Result:

Out of 618, 49 specimens were found significant with *Enterococcus faecalis* were found, 9 *Enterococcus faecalis* were resistant to fosfomycin (MIC  $\geq$ 512 µg/l), 04 strains were intermediates to fosfomycin (MIC=128 µg/l) and 36 strains were susceptible to fosfomycin (MIC  $\leq$ 64) as shown Table no. 2.

In our present study we had developed a practical disc potentiation test to an effectivity of sodium phosphonoformate with fosfomycin disc in the *Enterococcus faecalis* using PPF discs and found that no growth inhibition zone expanded near the fosfomycin discs with PPF as comparison to fosfomycin disc. Results, all *FREf* strains were no harboring any FRGST genes as shown in **image 5**.

**PCR:** For the identification and nucleotide sequencing of selected *FREf* strain (N=9), the PCR was performed to amplify the 16s rDNA and all bacterial DNA are 1496 bps were matched and proved that bacterial samples were *Enterococcus faecalis*. Further plasmid was

isolated from the obtained *FREf* strain and PCR against the *fosB* gene was performed. Obtained sequences 312 bps were matched with positive control of *fosB* gene and *FosB* gene expressed in all selected *FREf* which are responsible for the fosfomycin resistance capability. Obtained sequence used in BLAST and which is showing homology with plasmid belong to *Enterococcus faecalis*.

### Discussion:

Urinary tract infection is most common infection in the community and hospital settings. Fosfomycin works as an honest activity against the *Enterococcus* isolates infections and is employed traditionally in clinical settings worldwide <sup>(12)</sup>. The resistance rate of fosfomycin on *Enterococcus faecalis* was 18.4% in our present study whereas in another study *Enterococcus faecium* was 1.3% within the USA, 1% in south India, 4.9% and *Enterococcus faecalis* 0.3% in China documented <sup>(2)</sup>.

In the current study, we had done disk potentiation test of 9 *Enterococcus faecalis* which were resistant to fosfomycin (MIC  $\geq$ 512  $\mu$ g/l) and found that the absence of FR-GSTs in *FREf*. No-effectivity was observed of the sodium phosphonoformate with fosfomycin disc against the *FREf* – *fosB* gene. While in previous study, the good effectivity of PPF disc was observed in FosA3, FosA4 and FosC producing *Escherichia coli* and was positive for FR-GSTs <sup>(13)</sup>. Results, *FREf* does not produce FRGSTs as *E. coli*.

In our study, all 9 *FREf* strain harboured the *fosB* gene, which is to blame for the fosfomycin resistance. This may be delivered by plasmid mediated fosfomycin resistance genes or chromosomal mutations in various bacterial species, mutations in MurA are responsible to scale back the affinity with fosfomycin and results from inactive fosfomycin activities. In our study all, 9 *FREf* strains harboured *fosB* gene <sup>(14)</sup>.

We took *FREf* strains of *Enterococcus species* (N=9) and performed PCR analysis for the *fosB* gene to work out its expression that had potentially resulted within the resistant phenotype of *Enterococcus faecalis* as reported during this study. Previously, several mechanisms are introduced to related to fosfomycin drug resistance e.g. development of an passive adduct, mutation and enzymatic changes of fosfomycin within the target enzyme MurA that decrease the affinity of enolpyruvyl transferase enzyme for fosfomycin. Beside mutation in chromosomal bacterial genes encoding fosfomycin transporter are involved in resistance mechanisms. In past few reports, The FosB gene was mentioned as fosfomycin resistance gene *Enterococcus faecium* strains <sup>(2, 15, 16, 17, 18, 19)</sup>.

However, studying and validating mechanisms of *fosB* resistance was beyond the scope of this study since the only real detection of the *fosB* gene might be done during this research work and hence we encourage future proposals to work out the mechanism of resistance under the identical study design. We pursued detection and amplification of *fosB* gene in *E faecalis* cultures, by extracting DNA followed by performing PCR targeting 16S rDNA and *fosB* gene within the given bacterial isolates. Identification of bacterial species *Enterococci* isolated from clinical samples is taken in to consideration necessary, as is quantitative evaluation of their resistance to numerous antibiotics including among others vancomycin, teicoplanin, penicillin, ampicillin and high level resistance to gentamicin streptomycin <sup>(20)</sup>. It is also need to inform apart the low virulence *Enterococcus* with low level resistance to

antibiotic vancomycin from the species that is more frequently isolated from the clinical specimens, like *Enterococcus faecium* and *Enterococcus faecalis*, which in some countries can generally show high level transmissible and inducible resistance to glycopeptide <sup>(21)</sup>. One of the major suspicions in microbiology research is the correct identification of bacterial species. Without high level result output, accurate identification of target pathogens, studies in such important field as antibiotic resistance assessment, epidemiology and source of microbial tracking are compromised in the beginning. The aim of the protocol in this study was not definitive identification of each *Enterococcus species* present; rather it was progress of an efficient means of identifying a specific type of *Enterococcus species* which is *E faecalis*. Hence like several other studies, we also used the 16S rDNA approach to identify the organism of interest in our study <sup>(22, 23, 24)</sup>. As consistent with the results reported elsewhere, when amplified 16S rDNA gene and run on gel electrophoresis, we got a single band at the designated range of 1496 bps followed by sequencing thereby approving our approach of molecular identification of *Enterococcus faecalis* in the culture isolates used in this study.

Next, the *fosB* gene was targeted by designing specific sets of primers and amplifying them using a real-time PCR platform from Biorad (USA). The PCR product was electrophoretically analyzed and sequenced to match with *fosB* gene using BLAST. The results obtained confirmed the expression of *fosB* in the strains of *E. faecalis* studied, owing to the resistant phenotype exhibited by these organisms. Fosfomycin has a bactericidal action that inhibits the biosynthesis of peptidoglycan in both Gram-positive and - negative bacteria in the first step leading to destroying bacterial cell and death <sup>(3)</sup> Fosfomycin is a broad spectrum antibiotics and act as a phosphoenopyruvate (PEP) analogue and binds to enzyme MurA (UDP-GlcNAcenolpyruvyl transferase), an important accelerator for peptidoglycan synthesis <sup>(25)</sup> catalyzing the transfer of the enolpyruvylhalf of liveliness to the 3 $\phi$ -hydroxyl group of UDP-N-acetylglucosamine (UNAG) <sup>(26)</sup>. The alarming increase in antibiotic resistance together with that of Fosfomycin has inspired researchers to unendingly survey its molecular characteristics to raised perceive this potential drug for treating varied infectious diseases in India and round the world. Microorganism resistance to fosfomycin are often either body or plasmid-mediated. Most chromosomally resistant mutants that don't simply transfer to alternative organisms have associate degree impaired uptake system <sup>(27,28)</sup>, whereas plasmid-resistant mutants square measure usually proverbial to be multi-resistant and might transfer their resistance to alternative organisms through conjugation or transformation <sup>(14)</sup>. Irrespective of the mechanism, it's additional scary that the strains are becoming proof against such a potent antibiotic since this will limit treatment choices for UTI and alternative infectious diseases in close to future. However, todevelop simpler antibiotics, it's critically vital to unravel the molecular mechanism behind the resistant nature as exhibited by *E. faecalis* within the current study.

### **Conclusion:**

*FosB* gene is responsible for fosfomycin resistance in *Enterococcus faecalis* and could be transferred between bacterial strains. There are many studies done on the fosfomycin drug

susceptibility and resistance mechanisms but still not completed. So, further, must urgently more research on fosfomycin resistant mechanism.

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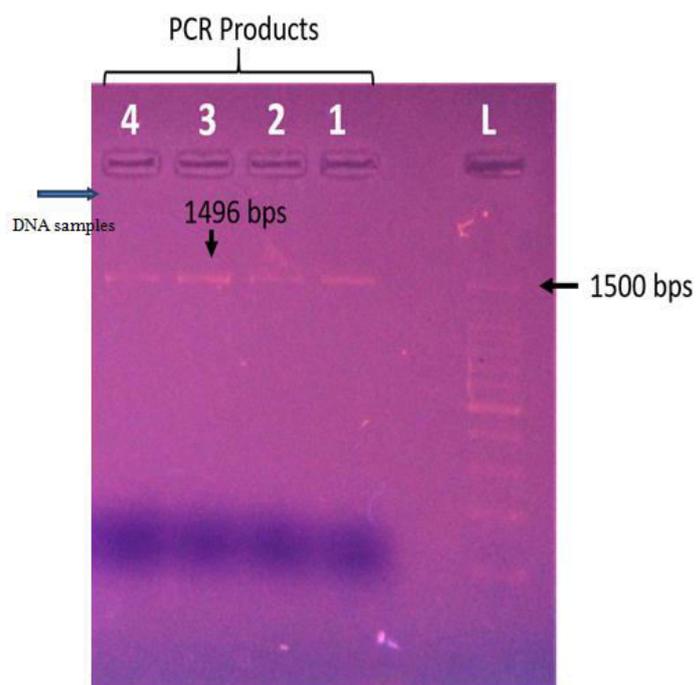


Figure 1 Gel Electrophoresis of bacterial DNA *Enterococcus faecalis*

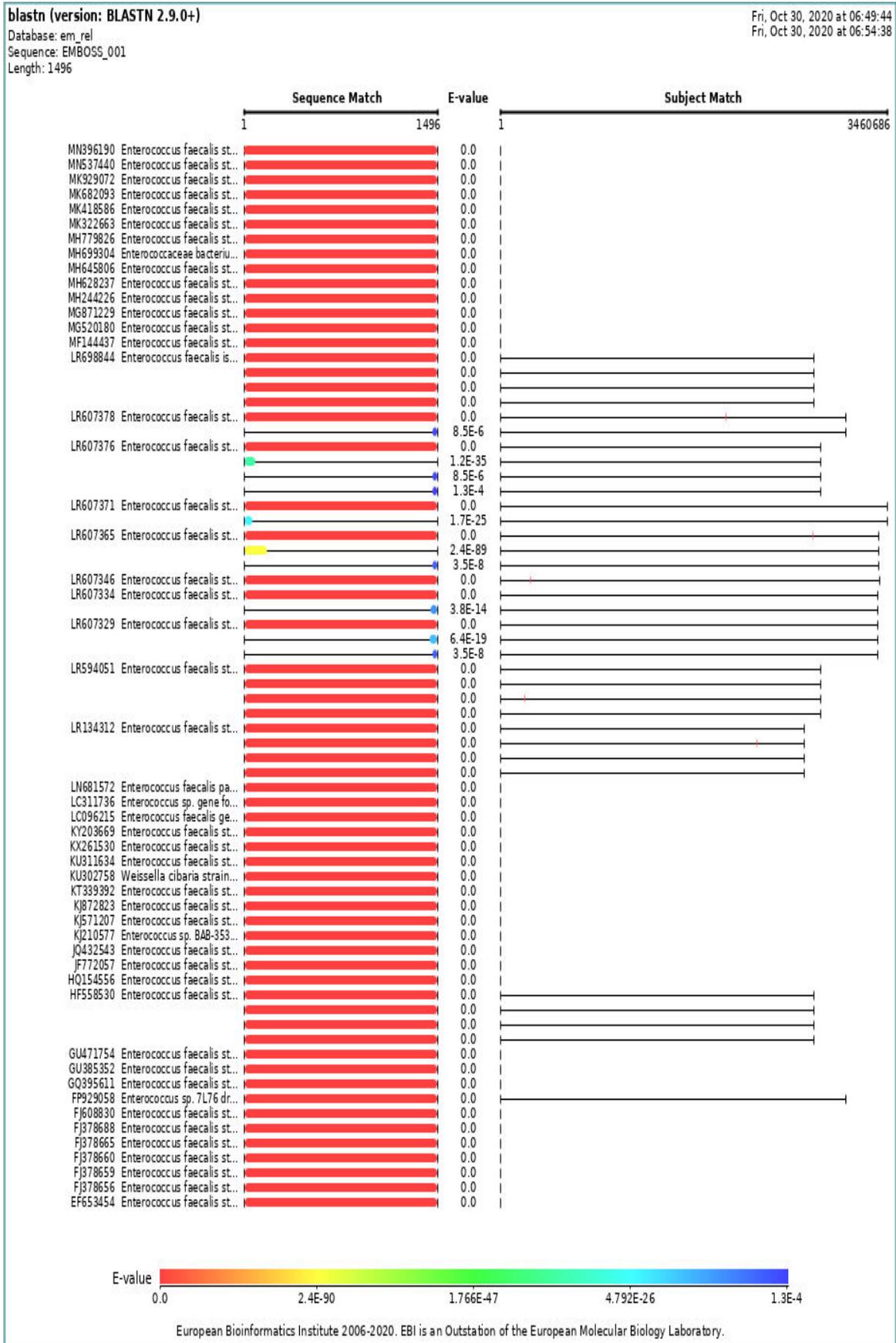


Figure 2 PCR sequencing 16S ribosomal RNA gene (1496bps)

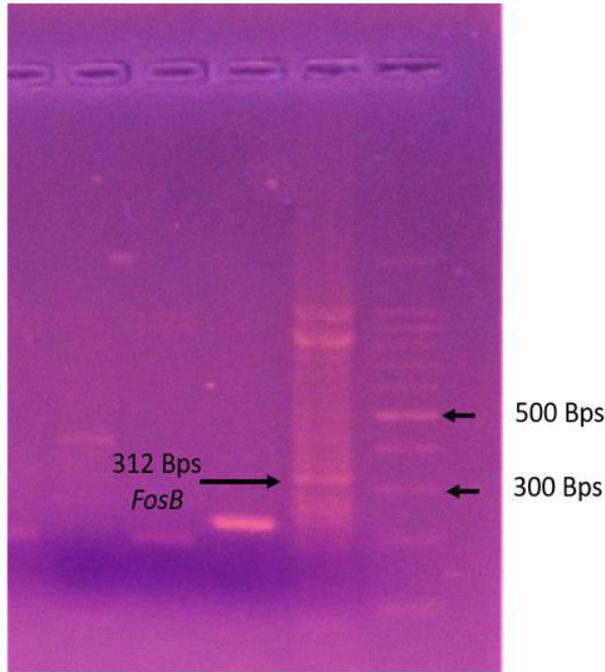


Figure 3 Gel electrophoresis of bacterial strain *FosB* (312 bps)

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**Enterococcus faecalis strain ES-1 plasmid unnamed2, complete sequence**

Sequence ID: [CP049777.1](#) Length: 53121 Number of Matches: 1

Range 1: 9853 to 10164 [GenBank](#) [Graphics](#) 
[Next Match](#)
[Previous Match](#)

Score	Expect	Identities	Gaps	Strand
577 bits(312)	2e-160	312/312(100%)	0/312(0%)	Plus/Plus
Query 1	CTCAATCTATCTTCTAAACTTCCTGTATGCAATTCTAATTTATGACCATCTAAATCAGTG	60		
Sbjct 9853	CTCAATCTATCTTCTAAACTTCCTGTATGCAATTCTAATTTATGACCATCTAAATCAGTG	9912		
Query 61	AAATATATTGATTTTTATCTCTAATATCTCTCTACCTCAAGAAATTTACTTCA	120		
Sbjct 9913	AAATATATTGATTTTTATCTCTAATATCTCTCTACCTCAAGAAATTTACTTCA	9972		
Query 121	TTTTCTTCAACCAGTTATACCAATCTCAAAATCGTTATCTGAAATTTGAAATGCTATA	180		
Sbjct 9973	TTTTCTTCAACCAGTTATACCAATCTCAAAATCGTTATCTGAAATTTGAAATGCTATA	10032		
Query 181	TGAGTATACGAATATTTAATTCGCTCTAGGAATAATTTTTCTCGTTCAAAGCTAAC	240		
Sbjct 10033	TGAGTATACGAATATTTAATTCGCTCTAGGAATAATTTTTCTCGTTCAAAGCTAAC	10092		
Query 241	CATATACCACTAAATTAATAATAGGCCAAGGCTCACTTCAACTAAATGTCAGCCCT	300		
Sbjct 10093	CATATACCACTAAATTAATAATAGGCCAAGGCTCACTTCAACTAAATGTCAGCCCT	10152		
Query 301	AAAATATCTCTG	312		
Sbjct 10153	AAAATATCTCTG	10164		

Figure 4 *FosB* gene (312 bps) sequencing

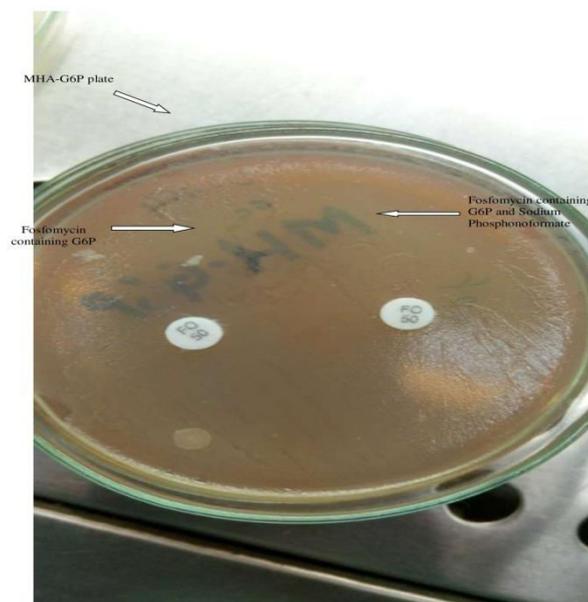


Figure 5 Disc Potentiation Test

Table 1 Primers sequence used in this study

Target gene	Used Primers	Size	Reference
16S rDNA R 16S rDNA F	‘5-CCCCGTCAGACGAACGCTGGC-3’ 5'-GACTTCACCCCAATCATCTATCCCACC-3'’)	1500 bps	Johnson, J.S et. al., (2019)
FosBF FosBR	5'-CAGAGATATTTTAGGGGCTGACA-3’ 5'-GACTTCACCCCAATCATCTATCCCACC-3’	312 bps	Xiucan Zhang et. al. , (2020)

Table 2 MIC of Fosfomycin on fosfomycin (50µg/ml) resistant

Fosfomycin resistant strains	Fosfomycin (MIC, 0.064-1024 µg/ml, Hi-media)								Total		
	8	16	32	64	128	256	512	>1024	S	I	R
<i>Enterococcus spp</i> (n=49)	00	01	07	28	04	00	03	06	36	04	09

S= Suseptible, I= Intermediate, R- Resistant