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Exposure to Biocides Leads to Ampicillin Resistance in Clinically Isolated *Enterococcus faecalis*: A Preliminary Study

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ABSTRACT

Background: Antibiotic resistance is one of the most urgent threats to the public health globally. The extensive use of biocides in clinical settings poses risk for emergence and spread of antibiotic resistance among nosocomial pathogens. This study aimed to investigate the effect of biocides exposure on the antibiotic susceptibility patterns, morphology and ultrastructure of clinically isolated *Enterococcus faecalis*.

Methods: The antibiotic susceptibility patterns of *Enterococcus faecalis* following biocides exposure were studied using disk diffusion and broth microdilution methods. Polymerase Chain Reaction (PCR) amplification was performed to confirm the ampicillin resistance genes. The effect on the morphological and ultrastructural of *Enterococcus faecalis* was characterized using transmission and scanning electron microscopy. All descriptive data from this study were presented as percentages.

Results: Exposure to subinhibitory concentration of hydrogen peroxide increased the MIC of *Enterococcus faecalis* EF2 and developed resistance to ampicillin. However, the exposure did not exhibit any effects to the ultrastructure and morphology of *Enterococcus faecalis*. Molecular analysis of *Enterococcus faecalis* EF2 and ampicillin resistant EF2 which was referred as EF2r in this study showed that both isolates contained pbp4 gene. Sequence analysis revealed that there was no mutation detected within the pbp4 gene thus suggesting that ampicillin resistance observed following exposure to subinhibition concentration of biocides did not cause any mutation within the pbp4 gene of *Enterococcus faecalis* EF2r.

Conclusion: This study demonstrated that exposure to subinhibitory concentration of biocides leads to reduced antibiotic susceptibility of the clinical *Enterococcus faecalis*. This investigation provides an insight into the event that could lead to the development and possible dissemination of antibiotic resistance determinants among nosocomial pathogens.

Keywords: General microbiology; Biocides; Antimicrobial susceptibility patterns; *Enterococcus faecalis*; pbp4 gene; Ampicillin resistant; Mutation

INTRODUCTION

Antimicrobial resistance is a global problem and the resulting risk for a number of serious infections and ineffective treatments. AMR could lead to 10 million deaths a year by 2050, and the world is expected to lose USD 100 to USD 600 trillion worth of economic output, between now and 2050 [1]. New resistance mechanisms are emerging rapidly, thus leading to an increased cost of healthcare with higher morbidity and mortality rates. In hospital settings, the increasing incidence of infections caused by antibiotic resistant pathogens is often resulted from selection of resistant mutant strains from the patients normal flora during antibiotic therapy or due to the conjugal transfer of mobile genetic elements (plasmid and transposon) [2].

In Malaysia, out of 6590 resistant isolates reported from January 2016 to June 2016, 2983 infections have been caused by resistant bacteria (ministry of health Malaysia, 2017). The National Surveillance of Antibiotic Resistance (NSAR) also reported an increase in vancomycin resistance among enterococci rising from 8.7% in 2012 to 14.9% in 2016 [3]. Currently, antibiotics of choice for enterococcal treatment include ampicillin, penicillin and vancomycin.

Biocides are broad spectrum antimicrobial chemicals that inhibit the growth of or that kill microorganisms. Ethanol, sodium hypochlorite, hydrogen peroxide and chlorhexidine are examples of commonly used disinfectants in clinical settings for preventing the transmission of hospital-acquired infections [4]. However, the efficiency and efficacy of biocides in eliminating bacterial contaminants have been a recurrent topic over the years owing to an increased possibility of cross-resistance to other biocides and antimicrobials. Exposure to low concentrations of biocides has been shown to significantly increase the transfer frequency of conjugative element, Tn916 that carry tetracycline resistance gene [5]. As Tn916 is the prototype of a large family of conjugative transposons described so far [6], this finding therefore highlights the risk of biocides exposure on the spread of other Tn916-like family and their resistance genes, which are found in an extremely diverse range of bacteria.

Genetic transfer causes alteration to the bacterial proteins, which can lead to antimicrobial resistance. In enterococci, mechanisms of ampicillin resistance have been described as the production of β-lactamases that cleave β-lactam ring of ampicillin molecule and the overproduction of Penicillin-Binding Proteins (PBPs) or alteration of Penicillin-Binding Proteins affinity [7,8]. Ampicillin targets and binds to specific low affinity PBPs leading to impaired cell wall synthesis and cell death [9]. Thus, alteration of PBPs affinity is the significant cause to β-lactam resistance (pbp4 in *Enterococcus faecalis* and pbp5 in *Enterococcus faecium*) [10]. Other than overproduction of PBPs, ampicillin resistance in *Enterococcus faecalis* and *Enterococcus faecium* were shown to be derived from the upregulated expression and amino acid substitution of PBP4 and PBP5 [11-14]. Point mutation in enterococcal pbp gene (PBP4 in *Enterococcus faecalis* and PBP5 *Enterococcus faecium*) can lead to high-level ampicillin and imipenem resistance [15]. The aim of the present study was to investigate the effect of biocides exposure to the antibiotic susceptibility patterns of clinical *Enterococcus faecalis* isolates and analysis of their ultrastructural changes following the exposure.

MATERIALS AND METHODS

Bacterial isolates

Clinical *Enterococcus faecalis* isolates that were previously collected from hospital Kuala Lumpur (HKL) Malaysia were used in this study (Table 1).

Table 1. *Enterococcus faecalis* isolates in this study.

Enterococcus faecalis	Characteristics
EF1	*Enterococcus faecalis* isolated from pus sample
EF2	*Enterococcus faecalis* isolated from blood sample
EF2r	*Enterococcus faecalis* EF2 that became resistant to ampicillin after exposure to biocide

Media, biocides and antibiotics

Brain Heart Infusion (BHI) and Muller Hinton (MH) agar and broth were from Oxoid, United Kingdom. Biocides that were used in this study included ethanol (100% v/v) (Sigma, Germany), hydrogen peroxide (50% v/v) (Sigma, Germany), sodium hypochlorite (50% w/v) (Sigma, Germany), and chlorhexidine digluconate (20% w/v) (Sigma, Germany). Antibiotics ampicillin (10 µg), vancomycin (30 µg), gentamicin (120 µg), teicoplanin (30 µg), linezolid (30 µg), and nitrofurantoin (300 µg) were purchased from Oxoid, UK.

Determination of biocides MICs

MICs of biocides were determined using broth microdilution method as recommended by the CLSI guidelines [16]. Isolates were grown on BHI agar plates for 18 hours-24 hours at 37°C. Colonies were suspended in 0.9% NaCl to an OD600 between 0.08 and 0.13 or turbidity equivalent to that of a 0.5 McFarland standard. The suspended colonies were then diluted to 100-fold in MH broth. Biocides from stock solutions were 2-fold diluted in 96-wells plate with 50 µl of biocide solution in each well. After the dilution series, 50 µl of biocide working solutions and 50 µl of cell suspension were pipetted into the 96-well microtiter plates and were incubated aerobically at 37°C for 16 hours-20 hours. The MICs were defined as the lowest concentration of the compounds that produce no visible growth of bacteria. Measurements were carried out in triplicates.

Exposure to sub-inhibitory concentrations of biocides

Isolates were exposed to sub-inhibitory concentration of biocides (determined as MIC/2) during mid-logarithmic phase (2 hours) incubation in (5 ml) MH broth. The optimum exposure time was determined using growth profiles (data not shown). The isolates were left to grow for another 2 hours at 37°C under shaking condition (180 rpm) prior to antibiotic susceptibility testing.

Antibiotics susceptibility testing

Antibiotics susceptibility testing were determined using disk diffusion and broth microdilution methods in MH agar and broth, respectively as recommended by CSLI, 2016. For disk diffusion, the inhibition zone was measured after 18 hours-20 hours incubation at 37°C. The broth microdilution was done in 96-wells plates [17]. Two-fold serial were prepared and inoculated plates were then incubated for 16 hours-20 hours at 37°C. MICs were determined as the

lowest concentration of antibiotics that produce no visible growth of bacteria as measured by microplate reader (Dynex MRX TC). Experiments were performed in triplicates and on three separate occasions.

Determination of bacterial morphology and ultrastructure

The morphology and ultrastructure of exposed and non-exposed isolates to biocides were observed using scanning (JEOL 6400 SEM) and transmission electron microscope (Hitachi H-7100 STEM). Specimens were prepared according to [18] and then dried in critical point dryer before coated with gold in sputter coater and viewed under scanning electron microscope. Prior to viewing under transmission electron microscope, the cells were sectioned (thick and ultrathin) using glass knife and ultramicrotome, followed by staining with uranyl acetate and lead stain. The cells were examined under 3500X-8000X magnifications.

Screening and detection of ampicillin resistance genes

Multiplex PCR amplification was performed to screen for genes responsible for ampicillin resistance using sets of primers as listed in Table 2. The PCR reaction mixture (25 µl) was prepared by mixing 12.5 µl of EconoTaq® PLUS GREEN 2x PCR master mix, 0.25 µl of both forward and reverse primers for each sample to make a total of 2 µl each, 1.0 µl of DNA template and 9.5 µl of RNase free water. The PCR protocol were as follows: Initial denaturation step of 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 52ºC for 1 minute, elongation at 72ºC for 2 minutes and a final elongation step at 72ºC for 10 minutes with 4ºC as holding temperature. All PCR products were purified and sequenced.

Table 2. Primers for PCR amplification of targeted genes.

Primer	Sequence (5'-3')	Amplicon	Size (bp)
Pbp5-F Pbp5-R	AGTGGCGGTTATTTTTACTA ACCGTCTGTATCTGTGAGGC	pbp5	900
BlaZ-F BlaZ-R	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	blaZ	173
pbp4 718F pbp4 1486R	TTTGTACCAATCACAGTTG CCCCCATCCGTAATGTTTG	pbp4	769
*pbp4 140F *pbp4 1132R	CAACGAAAGCCTGATGAAATGG AATCGCCTTTTTGAGGATCGG	pbp4 (confirmatory)	1272
*pbp4 1043F *pbp4 2130R	CGATTGACAGTGTACAACAACAAGC CGCTTCATTGTAGCACACTTTCCTTTTTC	pbp4 (confirmatory)	1087
Note: After screening by multiplex PCR, the presence of pbp4 gene in *Enterococcus faecalis* was further confirmed by single PCR amplification using the starred primers.			

Data analysis

The data analysis for the percentage of cultures with reduced susceptibility in antibiotics was done using statistical package (SPSS). The sequences were analysed using multiple sequence alignment (ClustalW) and BLASTn.

RESULTS

The MIC values and sub-inhibitory concentrations for *Enterococcus faecalis* isolates for each biocide are shown in Table 3. Sub-inhibitory concentration was taken as MIC/2.

Table 3. MICs and subinhibitory concentrations of biocides.

Enterococcus faecalis isolates	MIC[a]			
	EtOH (%)	HP (%)	SH (mg/ml)	CHX (µg/ml)
EF 1	1.82 ± 0.57 (0.91)	0.014 ± 0.004 (0.007)	0.775 ± 0.00 (0.388)	2.89 ± 1.05 (1.445)
EF 2	1.09 ± 0.00 (0.545)	0.013 ± 0.00 (0.007)	0.840 ± 0.38 (0.42)	2.00 ± 0.00 (1.00)

Note: EtOH=Ethanol; HP=Hydrogen Peroxide; SH=Sodium Hypochlorite; CHX=Chlorhexidine digluconate. [a]=Average of 3 independent experiments ± standard deviation.

Antibiotic susceptibility testing following exposure to sub-inhibitory concentrations of biocides

The antibiotic susceptibility profiles of *Enterococcus faecalis* isolates was studied by exposing the isolates to selected biocides (EtOH, HP, SH, CHX) for 2 hours. Table 4 summarizes the susceptibility results tested with six antibiotics (ampicillin, vancomycin, gentamicin, nitrofurantoin, linezolid, and teicoplanin) using disk diffusion method. The susceptibility patterns of some cultures (27.08%) either changed from susceptible to intermediate, intermediate to resistant or from susceptible to resistant following exposure to sub-inhibitory concentration of biocides. However, 72.92% of the cultures did not show any difference in the susceptibility patterns upon exposure to the biocides. There was not much difference in the percentage of reduced susceptibility for *Enterococcus faecalis* EF1 and EF2 with percentages of 25% and 29.17%, respectively as shown in Figures 1A-1C. All of the biocides had almost similar percentages of reduced susceptibility except for CHX (13.9%) with lower percentage. Among all antibiotics tested in this study, it was found that 83% of cultures showed reduction in susceptibility towards vancomycin post biocides exposure.

Figure 1. Percentage of cultures with reduced susceptibility after exposure to subinhibitory concentrations of biocides. **Note**: (A) Percentage of cultures with reduced susceptibility to antibiotics; (B) Percentage of cultures with reduced susceptibility to antibiotics based on the type of biocides; (C) Percentage of cultures with reduced susceptibility to antibiotics based on the type of antibiotics.

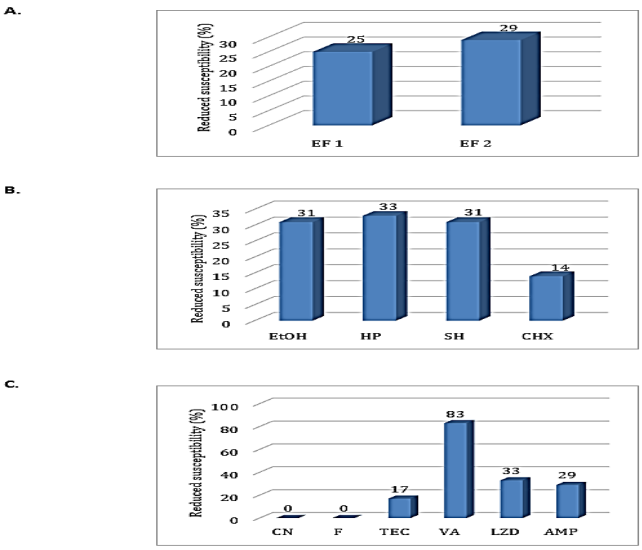


Table 4. Antibiotic susceptibility patterns before and after exposure to biocides.

Isolate	Condition		Set 1						Set 2						Set 3					
			CN	F	TEC	VA	LZD	AMP	CN	F	TEC	VA	LZD	AMP	CN	F	TEC	VA	LZD	AMP
EF1	STD		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	BE	EtOH	S	S	S	*I	S	S	S	S	S	*I	S	S	S	S	*I	*R	*I	S
		HP	S	S	S	S	S	S	S	S	*I	*I	*R	S	S	S	S	*I	*R	S
	AE	SH	S	S	S	*I	*I	S	S	S	S	*R	S	S	S	S	*I	*R	*I	S
		CHX	S	S	S	*I	S	S	S	S	S	*I	S	S	S	S	S	S	S	S
EF2	STD		R	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S	S
	BE	EtOH	R	S	S	*I	S	*R	R	S	S	*I	S	*R	R	S	S	*I	*R	S
		HP	R	S	*I	*I	*I	S	R	S	S	*I	S	S	R	S	S	*I	*I	*R
	AE	SH	R	S	S	*I	S	*R	R	S	S	*I	S	S	R	S	S	*I	S	*R
		CHX	R	S	S	*I	S	*R	R	S	S	S	S	S	S	S	S	S	S	*R

Note: BE=Before Exposure; AE=After Exposure; EtOH=ethanol; HP=Hydrogen Peroxide; SH: Sodium Hypochlorite; CHX=Chlorhexidine digluconate; CN=Gentamicin; F=Nitrofurantoin; TEC=Teicoplanin; STD=Standard value; VA=Vancomycin; LZD=Linezolid; AMP=Ampicillin; S=Susceptible; I=Intermediate; R=Resistance; The experiment was done in triplicates; *=The antibiotic susceptibility profiles that changed from susceptible to intermediate, susceptible to resistance, and intermediate to resistance.

MICs of *Enterococcus faecalis* isolates after exposure to sub-inhibitory concentrations of biocides

Following disk diffusion results, *Enterococcus faecalis* isolates were subjected to broth microdilution for MIC determination of antibiotics that have shown changes in the susceptibility patterns (ampicillin, vancomycin, teicoplanin, and linezolid). MICs were performed before and after exposure to sub-inhibitory concentrations of biocides. Results show that exposure to hydrogen peroxide have increased the MICs of ampicillin in both isolates (EF1 and EF2) from 1.95 µg/ml to 3.90 µg/ml and 1.62 µg/ml to 20.83 µg/ml, respectively (Table 5). Interestingly, the increased in MIC of ampicillin for EF2 has changed its antibiotic susceptibility pattern from susceptible to resistant.

Table 5. The MICs of selected antibiotics before and after exposure to subinhibitory concentration of biocides.

[a]Minimum inhibitory concentration (µg/ml)								
Biocide	EtOH		HP		CHX		SH	
	Before	After	Before	After	Before	After	Before	After
Ampicillin EF1	0.12 ± 0.00	0.12 ± 0.00	1.95 ± 0.00	*3.90 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00
Ampicillin EF2	1.62 ± 0.57	1.62 ± 0.57	1.62 ± 0.57	*20.83 ± 9.01	0.65 ± 0.28	0.98 ± 0.00	1.62 ± 0.57	1.62 ± 0.57
Vancomycin EF1	2.50 ± 0.90	3.13 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	2.61 ± 0.90	1.57 ± 0.00	1.31 ± 0.46
Vancomycin EF2	3.13 ± 0.00	4.17 ± 1.80	3.13 ± 0.00	2.61 ± 0.90	2.09 ± 0.90	3.13 ± 0.00	3.13 ± 0.00	6.25 ± 0.00
Teicoplanin EF1	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Teicoplanin EF2	0.02 ± 0.00	0.05 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00

Linezolid EF1	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.83 ± 0.07	0.63 ± 0.00	2.08 ± 0.02	0.63 ± 0.00	0.63 ± 0.00
Linezolid EF2	3.33 ± 1.44	3.33 ± 1.44	1.25 ± 0.00	1.04 ± 0.12	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	2.50 ± 0.00

Note: EtOH=Ethanol; HP=Hydrogen Peroxide; CHX=Chlorhexidine digluconate; SH=Sodium Hypochlorite; EF1 and EF2= *Enterococcus faecalis* isolates; [a]=Average of 3 independent experiments ± standard deviation. *=The MICs that increased after exposure to subinhibitory concentration of biocide.

Morphological and ultrastructural observations

Scanning electron microscopy was used to examine the ultrastructural changes of *Enterococcus faecalis* (EF2) resulted from exposure to sub-inhibitory concentration of hydrogen peroxide. The SEM analysis revealed that there was no visible effect observed in the exposed isolate as compared to the non-exposed isolate (Figures 2A and 2B). There was no sign of cell wall disruption such as cracks or holes observed. The exposed cells were intact with no deformities as there were no appearance of buds, grooves and bump on the cells surface. Furthermore, the exposed cells displayed no obvious difference in terms of length and width of the cells relative to the control cells (non-exposed). Transmission electron microscope allows the observation of inner structure of ultrathin sections by transmitting the electron beam across the thin sliced cells. Exposure to sub-inhibitory concentrations of hydrogen peroxide produced no visible changes in the spacing and thickness of the cell envelope in the exposed as well as the non-exposed cells (Figures 3A and 3B). There was no leakage of cytoplasmic constituent out of the cells as the cell wall was still intact displaying that no cell lysis occurrence.

Figure 2. Representatives of scanning electron micrographs of exposed and non-exposed *Enterococcus faecalis* EF2 to hydrogen peroxide. **Note**: (A) Non-exposed EF2 isolate; (B) Exposed EF2 isolate.

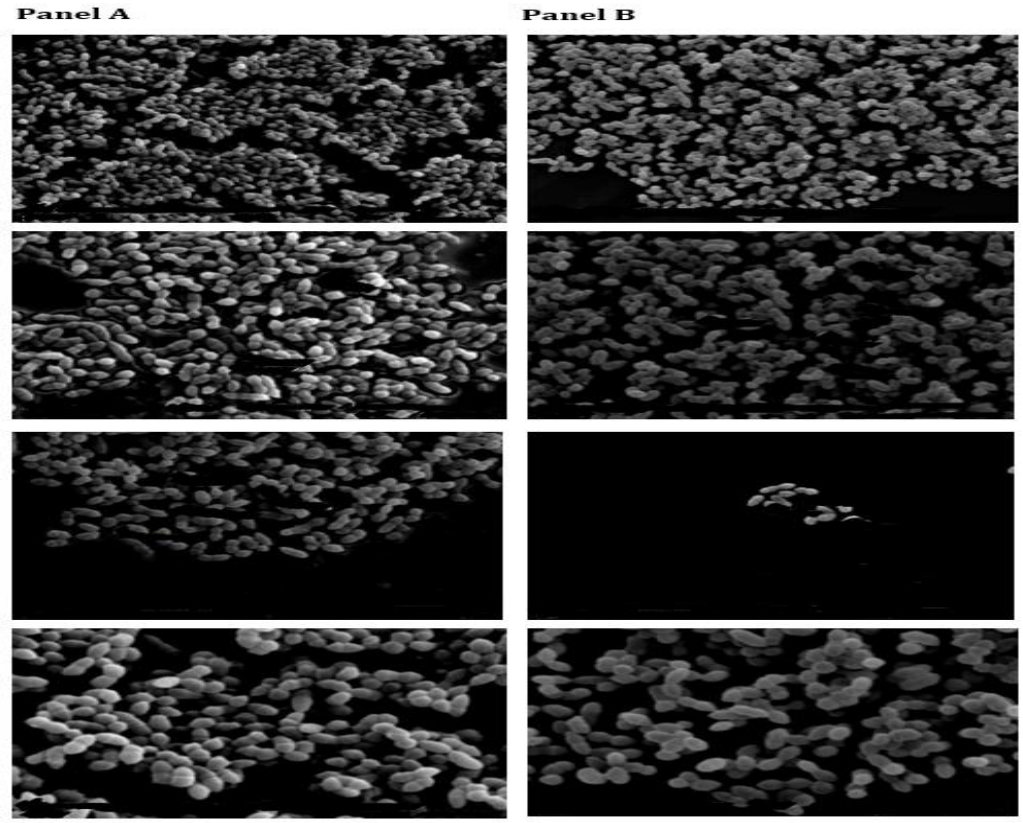
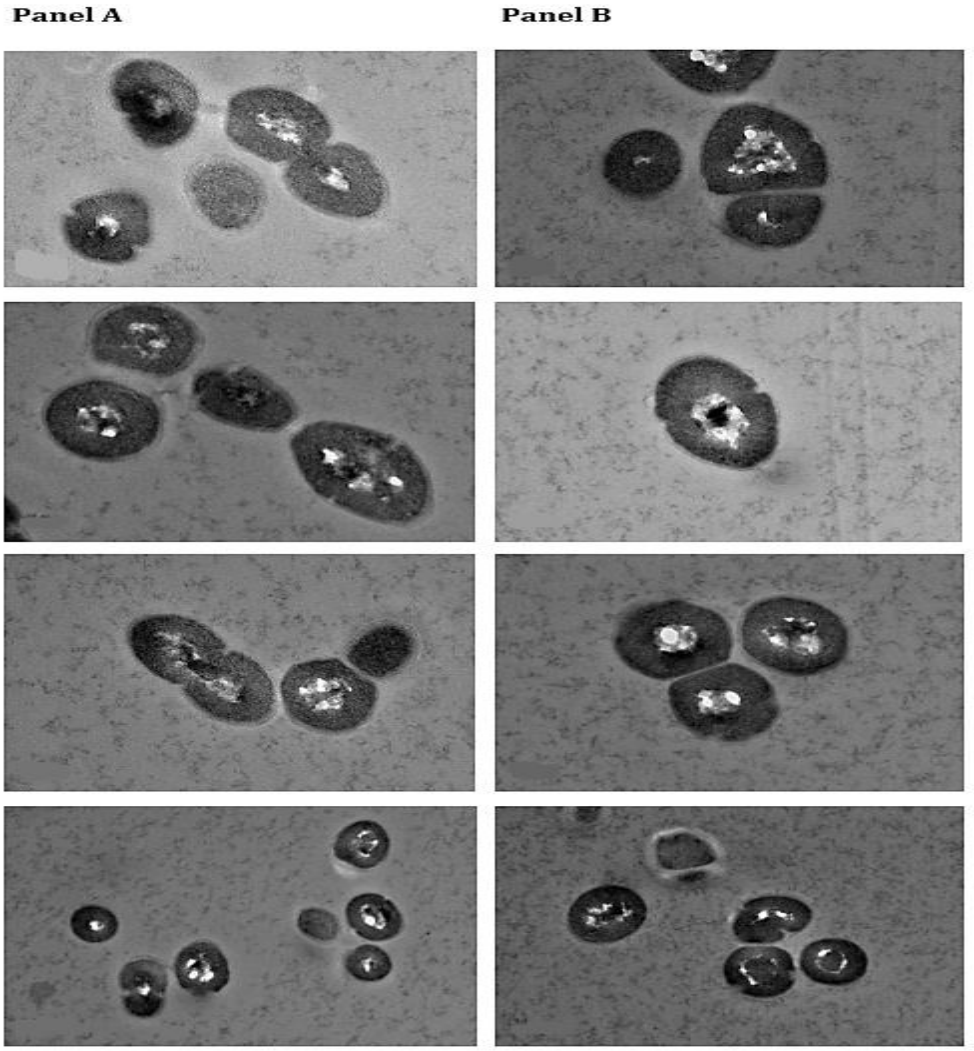


Figure 3. Representatives of transmission electron micrographs of *Enterococcus faecalis* EF2, before and after hydrogen peroxide exposure. **Note:** (A) Non-exposed *Enterococcus faecalis* EF2 isolate; (B) Exposed *Enterococcus faecalis* EF2 isolate.



The pbp4 gene conferred resistance to ampicillin

PCR screening showed that both *Enterococcus faecalis* EF2 and EF2r isolates harboured pbp4 gene that confers resistance to ampicillin. Analysis of the sequence showed a fully aligned sequence, with no mutation observed within the gene after exposure to sub-inhibitory concentration of biocide (hydrogen peroxide).

DISCUSSION

Biocides that were used in this study (ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite) are commonly applied in healthcare settings as disinfectants to prevent microbial contamination. Previous studies showed that enterococcus have MIC values to chlorhexidine digluconate between 2 µg/ml to 3 µg/ml and these values are in agreement with the MICs observed in this study [19]. However, there is a study that showed higher values of biocides MIC for *Enterococcus spp*. [20]. The differences in the MIC values might be contributed by the species and origin (food, clinical, animal, and environmental) of the enterococci isolates.

Enterococcus spp. has an ability to gain resistance toward antibiotics and its prevalence on multi drug resistance. However the data on the effect of biocides exposure on enterococcus resistance are still scarce. It has been reported that adaptation to biocides can reduce the susceptibility of bacteria towards other biocides and antibiotics

[17,21,22]. In this study, it was found that the susceptibility of *Enterococcus faecalis* to the antibiotics is reduced following exposure to sub-inhibitory concentrations of biocides. The reduction in susceptibility to antibiotics might be caused by expression of certain defense mechanism in bacterial cells resulting from compounds sub-lethal stress [23]. Mechanism of efflux in microbial cells also becomes a significant cause of bacterial resistance towards antimicrobial agent [24,25]. Environmental stress due to biocides exposure might alter the efflux mechanism in microbial cells resulting in an increase in antibiotic resistance. Interestingly, the MIC of ampicillin for EF2 increased and even crossed the resistance range after exposure to sub-inhibitory concentration of hydrogen peroxide. This result is in agreement with another study [26] in which an overnight exposure to sub-inhibitory concentrations of sodium hypochlorite and didecyldimonium chloride has significantly increased the MICs of *Pseudomonas aeruginosa* isolates to several antibiotics (amikacin, gentamicin, meropenem, ciprofloxacin).

Even though exposure to sub-inhibitory biocides affects the bacteria susceptibility pattern towards antibiotics, the morphology and ultrastructure remain unchanged. Sub-inhibitory concentration could be too low to exert any effects on the bacterial cell structure thus explain the ability of bacteria to survive and still grow under such exposure.

One of the *Enterococcus faecalis* isolate (EF2) became resistance to ampicillin when exposed to sub-inhibitory concentration of hydrogen peroxide. Ampicillin is a broad spectrum antibiotic that contain beta-lactam ring in its molecular structure. Mechanism of ampicillin resistance in enterococci has been reported to be caused by beta-lactamase production and overproduction of penicillin-binding protein [13]. Therefore, *Enterococcus faecalis* isolate that became resistance to ampicillin (EF2r) was screened for beta-lactamase gene (blaZ) and penicillin binding protein gene (pbp4 and pbp5). The pbp4 gene was detected in both EF2r and EF2 isolates. Amino acids substitution in pbp4 gene can alter the affinity of this protein that causes resistance to beta-lactam antibiotics. The pbp4 sequence revealed that there is no amino acid changes detected within the gene. However, it is suspected that although the stress from subinhibitory exposure of biocides did not cause any mutation in EF2r pbp4 gene, it might somehow alter the expression level as demonstrated in previous studies. Exposure to sub-inhibitory concentration of biocides has proven to increase β-glucuronidase enzyme activity as a crude assessment of transcriptional activity of tet(M) gene [5]. Other than that, a study demonstrated that reduced susceptibility in β-lactam antibiotics is significantly associated with increased expression and alteration of active site of *Enterococcus faecalis* pbp4 gene [27].

CONCLUSION

In summary, exposure to subinhibitory concentration of biocides can reduce susceptibility patterns to antibiotics. Such exposure can lead to an increase in resistance of nosocomial pathogens to antibiotics that are widely used for the treatments. Therefore, it is suggested that ampicillin resistance of EF2r might be due to alteration of pbp4 gene expression rather than amino acid substitution that changes the affinity of pbp4 protein causing resistance to ampicillin. Further investigation focusing on the expression of pbp4 gene following exposure to subinhibitory concentration of biocides is therefore necessary.

DECLARATIONS

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and materials

Please contact author for data requests.

Competing interest

The authors declare that they have no competing interests.

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Authors contributions

ASJ, RI and SKA designed the study. AJ, AFMN and NANMG drafted the manuscript. AFMN performed data analysis. All authors provided intellectual input to the study and read and approved the final manuscript.

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