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Effect of sub-minimum inhibitory concentrations of biocides on the selective pressure towards antibiotic resistance of *Staphylococcus aureus* strains

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ABSTRACT

Increased resistance of bacteria to antiseptics and disinfectants is not yet a significant problem, the issue as to whether low-level resistance to these agents is a selection factor for antibiotic-resistant strains in the clinical and domestic environments has yet to be settled. All isolates of *S. aureus* strains isolated from different clinical specimens submitted to the Microbiology Laboratory at Aljala Hospital, Benghazi, Libya of July 2019 were submitted to antibacterial susceptibility test before and after were exposure sub-minimum inhibitory concentrations (sub-MIC) of two biocides (ethanol 96% and sterillium). The results showed the MIC of biocides used in this study were not similar between the parent and daughter strains of the examined isolates, that the use of biocides may exert increased selective pressure on bacteria to acquire antibiotic and biocide resistance. The results of *S. aureus* after exposure to sub-MIC of ethanol 96% showed a multiple resistance to Erythromycin, Clindamycin and Cotrimoxazole, also the pattern of resistance was differed toward Vancomycin, but was not observed resistance to antibiotics after exposure (sub-MIC) of sterillium. These results suggest that exposure to sub-inhibitory concentrations of ethanol may lead to an increased selective pressure towards antibiotic resistance, which is clinically important since these disinfectants are frequently used in Benghazi hospitals.

Keywords: Sub-minimum inhibitory, biocides, selection of antibiotic, *Staphylococcus aureus*.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) remain a serious clinical threat and a leading cause of healthcare and community-associated infections worldwide. (Al-Abdli and Baiu, 2016; Tong et al., 2015). MRSA strains cause hard-to-treat infections because these are multi-drug resistant, such as beta-lactams, aminoglycosides and macrolides (Lyall, 2013) Exposure of bacteria to biocides can select for mutants with decreased biocide susceptibility that often display a decrease in susceptibility to antibiotics by intrinsic or acquired mechanisms of resistance (Painter et al., 2015)

Although widespread antimicrobial resistance (AMR) has been mostly attributed to the selective pressure generated by overuse and misuse of antimicrobials, concerns have been raised based on recent growing evidences regarding selection for AMR among bacteria exposed to non-antibiotic compounds used such as biocides used as disinfectants, antiseptics. Biocides (disinfectants, antiseptics, or preservatives) are widely used in healthcare or in the domestic settings in the community, it is now common to find biocides in cleaners, toothpaste, mouth rinse, cosmetics, an increasing trend towards a general and poorly directed use of biocides in the home environment for reducing microbial loads (Bal et al., 2006) Vitro studies suggest that exposure to sub-minimum inhibitory concentrations of biocides results in reduced susceptibility to antibiotics and biocides, the same survival strategies to disseminate acquired mechanisms of resistance to biocides as they have for resistance to antibiotics (Verraes et al., 2013; Russell, 2001).

There is no doubt that good hand hygiene reduces the risk of healthcare-associated infections. Worldwide, the most commonly used disinfectant or antiseptic agent is alcohol. Most alcohol-based hand antiseptics contain either ethanol, isopropanol or n-propanol, or a combination of two of these products. Due to its well-known antimicrobial properties, low cost and availability, therefore has become the active ingredient in most antimicrobial products for commercial use (e.g., hand sanitizers), increasing its popularity (Taskova, 2017). However, the use suboptimal concentrations may lead to the presence of sub-inhibitory concentrations; the bacterial response to sub inhibitory concentrations of biocides is unclear. The possible linkage between biocide and antibiotic resistance in bacteria and the role of biocides in the emergence of such resistance has provided more controversies in their extensive and indiscriminate usage (Gilbert and McBain, 2003).

It has been suggested *S. aureus* have adapted to biocide exposure by acquiring plasmids and transposons that confer biocide resistance. (Petersen, 2013) At present, insufficient scientific evidence exists to weigh these risks, and additional research is needed to allow appropriate characterization of risks in clinical and community environments.

The overall objective of this study was to examine if exposure to sub-antiseptics the selective pressure towards antibiotic resistance.

METHODOLOGY

Bacteria strains

This study involved eight *S. aureus* isolates from different clinical specimens submitted from the Microbiology Laboratory of Al jala Hospital, Benghazi, Libya of July 2019. Among 8 *S. aureus* isolates, 3 isolates were (MRSA). Identification of *S. aureus* were performed by traditional methods to study the phenotypic characteristics including gram staining, morphology, culture characteristics and biochemical reactions (Cheesbrough, 2009) cefoxitin DD test, and oxacillin DD test for detection of MRSA. Further, isolated strains were purified by streaking method.

Biocides

Two antiseptics were included in this study: Ethanol 96% and Sterillium (2-propanol; 1-propanol; ethyl-hexadecyl-dimethyl; ammonium-ethyl sulphate) were provided from the Department of Infection Control, medical Benghazi center. These 2 disinfectants were chosen because they are widely used in Benghazi Hospitals.

Determination MIC of antiseptics

Broth tube dilution method was used for determination of Minimum Inhibitory Concentration (MIC) of antiseptics against *S. aureus* strains. The broth dilution method described in this study is based on that described in the report of several studies (Ebrahimi et al., 2017; Balouiri et al., 2016; Jorgensen et al., 2015; Levinson and Jawetz, 2004) Briefly, the strains were revitalized on blood agar and then repassed to obtain a fresh, pure culture (Figure 1), colonies were resuspended in 0.9% NaCl to a turbidity equivalent to that of a 0.5 McFarland standard. It was determine the minimum inhibitory concentration (MIC)) for every antiseptics, through the classic method of successive dilution. In twelve numbered screw tubes (10 × 100 mm), 1 ml of MHb (Muller Hinton broth) medium was distributed for every tube, except for the tube number 1. The tubes were submitted to autoclave under constant pressure and temperature of 121°C. For the first and the second tubes of the series, 1 ml of tested antiseptics (Ethanol 96% and Sterillium) was added; tube 2 was stirred and 1 ml was withdrawn and transferred for tube 3. This successive transference was repeated until tube 11. It was added to all flasks, except for flask number 11, 0.1 ml of inoculation S.aureus . Incubate all tubes at 35°C overnight for 24 and 48 hours (Figure 2). Tubes 11 and 12 are positive (MHb + *S. aureus*) and negative (MHb + antiseptics) controls. The MIC was determined as the highest dilution of antiseptics that visually inhibited bacterial growth.

Figure 1. *S .aureus* strains on blood agar.

Exposure to subMICs of biocides

We used modification method was used by Alsaady and

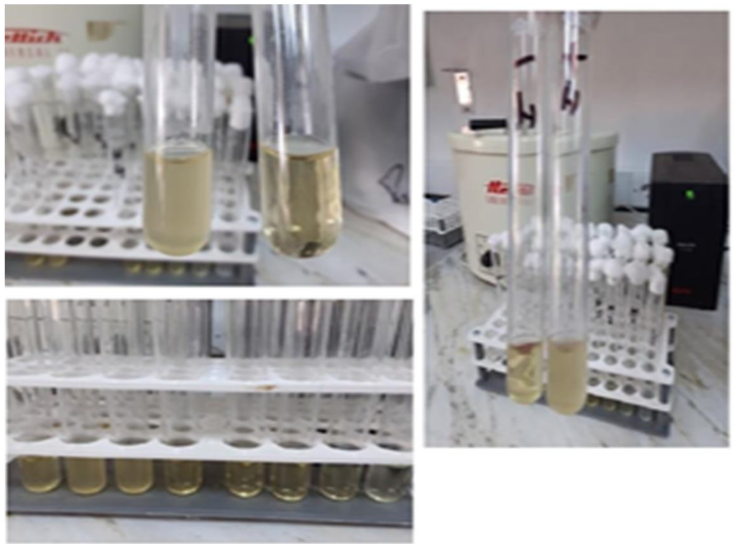

Figure 2. MIC of alcohol against *S. aureus* strains.

Ebrahimi (Alsaady, 2011; Bogdan et al., 2017; Ebrahimi et al., 2017). Briefly, the effect of sub-MICs of the previously mentioned biocides were performed by mixing of aliquots (2 ml) of bacterial suspensions with concentration of biocides (1:1) within 1/2, 1/4, 1/8 and 1/16 of previously determined MIC of biocides. Tubes were incubated at 37°C for 24 to 48 hours, followed by centrifugation for 5 minutes at 4500 rpm and washed once with washing buffer (1 mol l)1 NaCl, 10 mmol l)1 EDTA, pH 8Æ0). Samples from sediment of each tube were sub-cultured on Nutrient Agar (NA) plates containing four MICs of antiseptics and incubated as above. One colony with the typical size and morphology of the original strain was chosen randomly from each of the latter NA plates and sub-cultured onto antiseptics free Broth and incubated at 37°C for 24 hours. The daughter cells were examined for determination for antibiotics susceptibility by disk diffusion method Kirby-Bauer (Bauer et al., 1966) and a comparing the antibiotics susceptibility before and after exposure to antiseptics and MIC for the selected isolates was retested using the broth tube dilution method as previously described.

Antimicrobial susceptibility testing

The disk diffusion method before and after exposure to the antiseptics was performed according to the methodology described in the Clinical and Laboratory Standards Institute (CLSI-2016) guidelines. The tested antibiotics were as follows: meropenem 10 µg (Oxoid), Erythromycin 30 µg (Oxoid), Clindamycin 2 µg (Oxoid), Co-trimoxazole 25 µg (Oxoid), levofloxacin 5 µg (Oxoid), Gentamicin 10 µg (Oxoid), Ciprofloxacin 5 µg (Oxoid), Oxacillin 1 µg (Oxoid), Cefoxitin 30 µg (Oxoid) and Vancomycin 30 µg (Oxoid), (Figure 3) to identify antibiotic resistance after were exposure sub-minimum inhibitory concentrations (sub-MIC) of ethanol 96% and sterillium. Only antibiotic-susceptible or intermediate isolates were included.

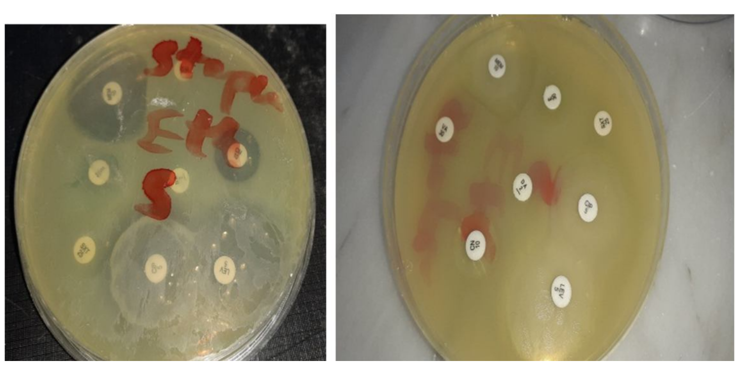

Figure 3. Disk diffusion susceptibility test (Kirby-Bauer test).

RESULTS

We tested the MIC of two antiseptic (ethanol 96% and sterillium) of a total of 8 *S. aureus* strains (3 MRSA and 5 MSSA) isolates from different clinical specimens submitted to the Microbiology Laboratory at Aljala Hospital, Benghazi, Libya of July 2019, this study showed:

For ethanol 96%, the MIC were (234.38 μg/ml) for all isolates (MRSA and MSSA) for the parent strains while, the MIC for two MRSA isolates were differed for the mutant strains (468.75 μg/ml); for sterillium, the MIC were (937.5 μg/ml) for all isolates (MRSA and MSSA) for the parent strains while, the MIC for two MRSA isolates were differed for the mutant strains were (1875 μg/ml) (Figure 4).

The antibiotic susceptibility pattern, using the disk diffusion method, of the *S. aureus* isolates is shown in Table 1.

The number and percentage of isolates that showed a multiple resistance to antibiotics after exposure (sub-MIC) of ethanol 96% are shown in Table 2. Briefly, a multiple resistance of the susceptible isolates were observed of Erythromycin in 2/6 (33.3%), Clindamycin in 2/5 (40%), Cotrimoxazole in 1/7 (14.3%), and Gentamicin in 1/8 (12.5%), as well as vancomycin in 1/8 (12.5%), but was not observed resistance to antibiotics after exposure (sub-MIC) of sterillium.

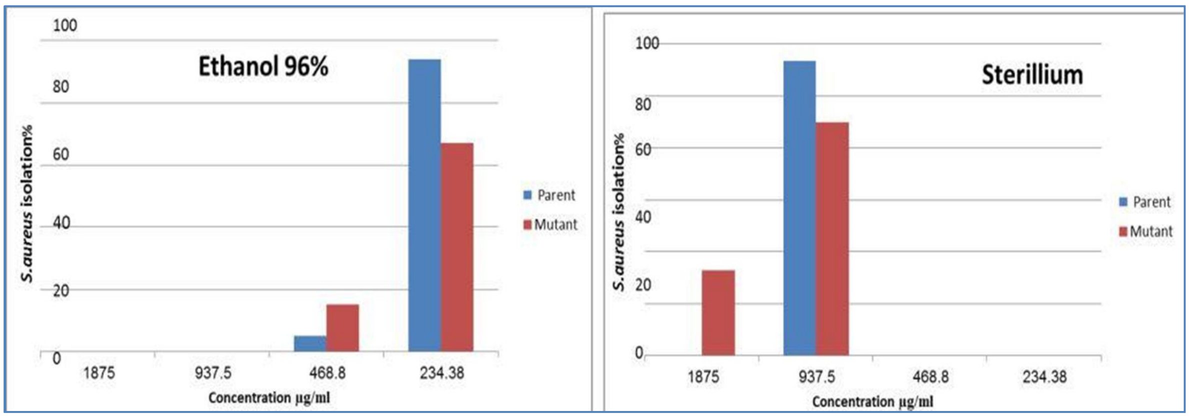

Figure 4. MIC of each (Ethanol 96% and Sterillium) for the parent strains and mutant strains of *S. aureus*, expressed as percentages.

Table 1. Antibiotic susceptibility pattern of the *S. aureus* isolates to the tested antibiotics before exposure to antiseptics.

Antibiotic(s)	Susceptible		Intermediate		Resistant	
	No	%	No	%	No	%
Vancomycin	8	100	**0**	**0**	0	0
Levofloxacin	8	100	**0**	**0**	0	0
Gentamicin	8	100	0	0	0	0
Ciprofloxacin	6	75	0	0	2	25
Erythromycin	6	75	0	0	2	25
Cotrimoxazole	7	87.5	0	0	1	12.5
Clindamycin	5	62.5	0	0	3	37.5
Meropenem	8	100	0	0	0	0
Oxacillin	5	62.5	0	0	3	37.5
Cefoxitin	5	62.5	0	0	3	37.5

DISCUSSION

Improved understanding is needed of the effectiveness of antiseptic and disinfection, to reduce the incidence of infectious diseases and colonization in healthcare workers and patients in Benghazi Hospitals, staphylococcus infection is a serious problem of hospital-acquired infections in various departments. In addition,

Table 2. Antibiotic resistance change before and after exposure to ethanol 96%.

Antibiotic(s)	Pre-exposure		Post-exposure	
	Sensitive	%	Resistance	%
Vancomycin	8	100	**1**	12.5
Levofloxacin	8	100	**0**	0
Gentamicin	8	100	Intermediate	12.5
Ciprofloxacin	6	75	0	0
Erythromycin	6	75	2	33.3
Cotrimoxazole	7	87.5	1	14.3
Clindamycin	5	62.5	2	40
Meropenem	8	100	0	0
Oxacillin	5	62.5	0	0
Cefoxitin	5	62.5	0	0

hospital-acquired infections caused by MRSA was common in Libyan Hospitals in the last decade, frequently detected in healthcare workers and patients, according to several studies from Libya (Ghenghesh et al., 2013; Al-Abdli and Baiu, 2014; Baiu and Al-Abdli, 2015; Al-Abdli and Baiu, 2016; BenDarif et al., 2016; Zawia and Al Habishi, 2017). The problem of antibiotic resistance is very serious in Libya and appears to be on the rise (Mathlouthi et al., 2014). High resistance rates were observed of MRSA that exhibit resistance to several classes of antibiotics, including beta-lactams, aminoglycosides and macrolides (Lohr et al., 2017).

There is evidence that pathogenic bacteria can adapt to antiseptics upon repeated exposure. (Verspecht et al., 2019) Some biocides are reported to share the same mechanism of action with some antibiotics, and this can cause resistance to disinfectants used in environmental cleaning. (Webber et al., 2015) More alarming is the concomitant increase in antibiotic resistance for some pathogens. Effects of sub-MICs of antibiotics or biocides on bacterial virulence factor expression may provide additional information for the rational use of antimicrobials in clinical practice. Therefore, this study aimed to examine if exposure to low concentrations to antiseptics the selective pressure towards antibiotic resistance.

This study showed that the MIC of ethanol 96% for all *S. aureus* isolates was 234.38 µg/ml for the parent strains. These figures are similar to those noted previously with other concentrations of ethanol for *S. aureus* isolates (Valle et al., 2016; Man et al., 2017). This MIC was much lower than that determined by Rebaya et al. (2016) and Jeyaseelan and Jashothan (2012); for sterillium, the MIC were (937.5 µg/ml) for all *S. aureus* isolates for the parent strains. In this study we showed the results showed the MIC of the ethanol and sterillium used in this study were not similar between the parent and daughter strains of the examined isolates. These results are similar to those noted previously with other antimicrobial and other species of bacteria in a previous studies (Ebrahimi et al., 2017).

The findings show that despite the ability of ethanol-based disinfectants of inhibiting the growth of *S. aureus* isolates in concentrations as low, as reported in the present study, it is questionable if these low concentrations can be safely recommended. In fact, the use of low disinfectant concentrations can be a crucial cause of the emergence of antibiotic-resistant *S. aureus* strains, Low level ethanol exposure reduced the susceptibility to selected antibiotics in *S. aureus* in this study.

The association between antibiotic resistance and exposure to disinfectants can be also attributed to the relevant effects of these antimicrobial agents on bacterial community structure. Increased resistance to antiseptics and disinfectants has been associated with mutation and/or presence of plasmids, and both have been observed in some strains of bacteria (Galhardo et al., 2007; Painter et al., 2015; Peterson et al., 2018). Some studies have also suggested a potential molecular link between reduced susceptibility observed in some disinfectants and antibiotic resistance (Nasr et al., 2018). In this study after exposure to sub-minimum inhibitory concentrations of ethanol a multiple resistance of the susceptible isolates were observed of Erythromycin, Clindamycin, Cotrimoxazole, Gentamicin, and vancomycin.

To our knowledge, no previous studies have examined the effect of exposure to these 2 biocides on *S. aureus* resistance to antibiotics; however, Many studies assessed that the use of biocides alone or combined with antibiotic treatment may exert increased selective pressure on bacteria to acquire antibiotic and biocide resistance, and their results regarding biocides effect was similar to ours (Mcdonnell and Russell, 1999; Alsaady, 2011; Nasr et al., 2018; Pando et al., 2017).

Conclusion

The results in this study suggest that exposure to sub-inhibitory doses of various biocides can induce antibiotic resistance pathogenic clinical isolates, which is clinically important since these disinfectants are frequently used in hospitals. Thus, the use of appropriate bactericidal concentrations of various biocides should be emphasized by the infection prevention and control specialist as a part of infection control program standards in healthcare settings.

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Conflict of interest

The authors declare that there is no conflict of interest.

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