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Susceptibility of Four *Bacillus Spp.* Recovered from Contaminated In-Use Antiseptics and Disinfectants to Antibiotics and Biocides. Any Significance?

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ABSTRACT
From contaminated in-use antiseptics and disinfectants collected from various places in Amman / Jordan community, during Corona-19 pandemic, thirty-two isolates of four Bacillus species: B. lentus (N=11), B. cereus (N=10), B. subtilis (N=9) and B. circulans (N=2) were recovered. These isolates were examined for their susceptibility to six antibiotics: Penicillin G (10IU), Gentamycin (10µg), Tetracycline (30µg), Erythromycin (15µg), Cefepime (30µg), Chloramphenicol (30µg) and Ciprofloxacin (5µg) and toward four biocides: 70% and 80% Alcohol, 10% Iodine and 0.13% Benzalkonium chloride (under clean and dirty conditions). Multiple resistance up to four antibiotics was detected by B. lentus. Heteroresistance toward one and four antibiotics was presented by B. lentus and B. circulans respectively. Simultaneously both Bacillus lentus and B. circulance exhibited resistance to all tested biocides under both clean and dirty conditions. Both 10% Iodine and 0.13% Benzalkonium chloride demonstrated the highest activity among other tested biocides though, dirty condition exerted marked effect in reducing biocides efficacy. Results demonstrated by B. lentus and B. circulans may signify cross-resistance to antibiotics and biocides, treatment failure and a probably increased community acquired infections following uncontrolled and misuse of biocides.
Keywords: *Bacillus, Biocides, Antibiotics, Cross-Resistance, heteroresistance*

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I. Introduction

Antiseptics and disinfectants are biocides, well known as the first line of defense against disease forming agents. They are widely used by European Union (EU) in healthcare settings, household and industry to destroy or inhibit disease forming microorganisms (1). Despite their lethal effects that may be exerted on humans, animals, the environment and ecological balance (2, 3); antiseptics are used to minimize the transitory microbes on the skins, specifically hands and mucous membranes among community individuals; whereas disinfectants are applied to nonliving surfaces to eradicate hazardous potential pathogenic microorganisms.

Worldwide pandemic was declared by WHO on January 2020 (4) succeeding the appearance of a new coronavirus at the end of 2019. The grave situation has promoted the implementation of preventative measures, the most important of which was the continuous use of antiseptics and disinfectants as essential mean for protection and to control transmission of the virus in the environment and among community population (5,6).

Nonetheless, incorrect societal behavior and misuses have emerged among members of the community, regardless of health organized directions of how to deal with antiseptics and disinfectants. These included: incorrect choice of a disinfectant, insufficient disinfection of environmental surfaces, inappropriate concentration of a biocide, inadequate contact time or contaminated disinfectant; one or more of these malpractices may lead to a decrease in the effective concentration of the active agents resulting in microbial resistance (non-susceptibility). A continuing and increasing concern whether bacterial resistance to biocides could contribute to selection of bacterial genera and species tolerant or less susceptible to the biocide or whether the biocide facilitates the selection of antibiotic resistant bacteria; may probably display cross-resistance to certain antibiotic which in turn will pose a serious health risk to the community (2,7,8) This selective pressure might result in increased emergence of antibiotic resistance, already occurring worldwide as the main reason for treatment failure (2, 9).

Generally, biocides have a broader spectrum of activity than antibiotics, and exert nonspecific action on microbial cells by employing action on multiple targets, while antibiotics have specific intracellular targets (10). Nevertheless, laboratory-based surveys have described that bacteria display possible common mechanisms conferring resistance to biocides and antibiotics (11,12). However, Jones and Joshi (1) indicated that biocidal activity utilizes unpredictable approaches to kill microorganisms, ranging from oxidization to solubilizing lipids.

Bacillus spp. and *Staphylococcus spp*.; the major causes of wide variety of human diseases, were the most frequent bacteria identified in contaminated biocides, in addition to Gram negative bacteria (13,14,15,16,17) The genus *Bacillus* is Gram-positive rod-shaped commonly found in soil, food, and marine sponges. Some species of *Bacillus* are toxin producers such as *B. cereus* and *B.lentus. B. circulans* is a soil-resident human pathogen, associated with septicemia, mixed abscess infections, and wound infections, as well as with meningitis; whereas *B. subtilis* is non-pathogenic for humans, is ubiquitous in the environment, normally found in soil and vegetation considered.

The purpose of the present study is to investigate susceptibilities of the above four *Bacillus* species recovered from contaminated in- use antiseptics and disinfectants collected from the community toward six antibiotics and toward four biocide agents under clean and dirty conditions to find out whether any correlation is reported in resistance against biocides that could contribute to resistance to antibiotics.

II. Materials and Methods:

From contaminated in-use antiseptics and disinfectants collected from various places in Amman / Jordan community during Corona-19 pandemic, thirty-two isolates of four Bacillus species: B. lentus (N=11), B. cereus (N=10), B. subtilis (N=9) and B. circulans (N=2) were provided by Al- Husami (18) and examined, using bacterial inoculum equivalent to 0.5 McFarland, alongside with the reference strains of B. cereus ATCC 10876 and B. subtilis ATCC 11774 for their:

1. Antibiotic susceptibility toward the following antibiotics, (Oxoid): Penicillin G (10 IU), Gentamycin (10µg), Tetracycline (30µg), Erythromycin (15µg), Cefepime (30µg), Chloramphenicol (30µg) and Ciprofloxacin (5µg). Kirby-Bauer disk diffusion method, was adopted according to the guidelines of the Clinical Laboratory Standard Institute (19) using Mueller-Hinton Agar (MHA) (HIMEDIA® M173). Inhibition zones were measured by millimeters and isolates were recorded as sensitive or resistant after interpreting the results with standard tables.

2. Biocides susceptibility toward four types of antiseptics and disinfectants by using the Kirby-Bauer disk diffusion method (19). These are: 70% and 80% Alcohol, 10% Iodine and 0.13% Benzalkonium chloride. The experiment was conducted under clean and dirty condition to mimic real situation. To provide dirty condition, 0.3% of yeast was added to each type of the above biocides (20). Blank disks were impregnated into indicated biocide solution by using sterile forceps (Dipped into spirit then flamed), disks were put on the inoculated MHA plates gently but firmly pressed onto the agar surface, then plates were incubated for 24 hrs. at 35°C. A ruler was used to measure the diameters of inhibition zones by millimeters. According to the diameter of inhibition zone, interpretive criteria was recorded as following: Resistant =10 mm or less, Intermediate =11-15 mm and Susceptible= 16 mm and above (21).

In both above experiments intermediate zones were included with the resistant ones

III. Results:

Table (1) shows susceptibility of four Bacillus spp. recovered from contaminated antiseptics and disinfectants toward six antibiotics. All Bacillus isolates were resistant to penicillin. B. subtilis was the most susceptible species toward 5 antibiotics followed by B. cereus (susceptible to 4 antibiotics). All isolates showed susceptibility toward: gentamycin and ciprofloxacin except *B. lentus*, and to cefepime except *B. cereus*. The species *B. subtilis, B. cereus* and *B. circulans* were susceptible to erythromycin.

Table (1) Antibiotic susceptibility of four *Bacillus spp* recovered from in-use samples of antiseptics and disinfectants

Antibiotics (µg) / Diameter (mm)	*B. lentus* (N=11)	*B. circulans* (N=2)	*B. cereus* (N=10)	*B. subtilis* (N=9)
Penicillin (10 units)	6 (R)	28(R)	19 (R)	27 (R)
Tetracycline (30)	19 (S) H	18 (R)	20 (S)	21 (S)
Erythromycin (10)	8 (R)	23 (S) H	24 (S)	29 (S)
Gentamycin (10)	13 (R)	16 (S) H	20 (S)	21 (S)
Cefepime (30)	19 (S)	20 (S) H	17 (R)	25 (S) H
Ciprofloxacin (5)	19 (R)	22 (S)	21 (S)	27 (S)

R= Resistance, S= Sensitive , H= Heteroresistance

Figure (1) demonstrates heteroresistance which is a substantial reduction in antibiotic susceptibility which was clearly exhibited by *B. circulans* toward three antibiotics. Also, heteroresistance was displayed by *B. lentus* and *B. subtilis* toward tetracycline and cefepime respectively.

Figure (1): Heteroresistance

Susceptibility of *B. cereus*, *B. circulans*, *B. lentus* and *B. subtilis* toward four active agents of antiseptic and disinfectant samples under clean and dirty conditions is illustrated in Table (2). According to the scale of the diameter of inhibition zone adopted from (21) for measuring effectiveness of biocides on bacterial growth. It is markedly evident that both *B. circulans* and *B. lentus* were completely resistant to all biocides under both clean and dirty conditions**.** Whereas *B. subtilis* displayed susceptibility toward all biocides in both clean and dirty conditions, apart from 10% Iodine and 80% alcohol in dirty conditions,

Table (2) Biocides susceptibility of recovered bacteria from contaminated in-use samples of antiseptics and disinfectants

Bacterial Isolates	10% Iodine		0.13% Benzalkonium chloride		Alcohol			
					80%		70%	
	Clean	Dirty	Clean	Dirty	Clean	Dirty	Clean	Dirty
B. circulans	**14 (R)**	**4 (R)**	**8 (R)**	**3 (R)**	**10 (R)**	**(R)**	**14 (R)**	**11(R)**
B. lentus	**10 (R)**	**8 (R)**	**8 (R)**	**3 (R)**	**8 (R)**	**6 (R)**	**10 (R)**	**4 (R)**
B. cereus	**15 (R)**	**10 (R)**	**19 (S)**	**14 (R)**	**17 (S)**	**12 (R)**	**27 (S)**	**19 (S)**
B. subtilis	**16 (S)**	**13 (R)**	**22 (S)**	**19 (S)**	**16 (S)**	**4 (R)**	**25 (S)**	**21 (S)**

S: Susceptible, R: Resistant, H: Heteroresistance., Resistant =10mm or less; Intermediate =11-5mm; Susceptible= 16mm

IV. Discussion

Significant health risk to the community could result due to microbial contamination of antiseptics and disinfectants, most important of which is the potential existence of a linkage between biocide usage and antibiotic resistance (22,23,24,25). The widespread and misuse of biocides such as exposing microbes to sub inhibitory concentration (26) and/or extensive use of biocidal product (27, 28) has provoked some assumptions on increased microbial resistance to antibiotics, specifically whether resistance is induced by antiseptics or disinfectants or acquired. However, the effect of biocides on the bacterial cell is complex and the emergence of bacterial cross-resistance following exposure to biocides might be strain specific rather than species or genus specific (29).

It was reported that exposing bacterial flora on human skin and those in the environment, repeatedly to certain biocides may lead to reduced susceptibility of certain microbes to specific biocides and can survive particularly if bacteria harbor resistance genes which may spread to other bacteria (25).

The risk of contaminations of most antiseptics and disinfectants can lead to changes in their physical and chemical properties in addition to reduced efficacy. A situation has led to outbreaks in nosocomial infections which mainly developed in hospitals and other care health setting. Results demonstrated by the present study on biocides collected from the community may predict probably a rise in community acquired infections, following the massive and uncontrolled use of biocides practiced during the period of Covid-19 pandemic, at home care settings, educational institution, markets or at workplaces (30).

Emerging bacterial resistance to biocides has been well described and reported in vitro with compounds such as: chlorhexidine quaternary ammonium compounds, bisphenol, triclosan, iodophore and even with high reactive biocides such as glutaraldehyde and peroxygens (25).

Most biocides are prepared as alcohol- based products; alcohols kill all vegetative microbial forms resulting in progressive and exponential decline in alcohol potency and efficiency. Ultimately, some species of bacteria are able to survive and adapt with the new environmental conditions (31). Besides, alcohols are not able to destroy bacterial spores which might contaminate these solutions (32). Spores of many species belong to the aerobic *Bacillus spp*. and to less extent the anaerobic *Clostridium spp.* are extremely resistant; they can survive and persist for long periods in contaminated biocides (33). Accordingly, spores can be germinated when biocide efficacy is reduced; making the situation even worst.

Zones of inhibition of *Bacillus spp.* were interpreted using *Staphylococcus spp*. breakpoints because there are no standard antibiotic disk diffusion resistance breakpoints defined for *Bacillus spp* (34). Also, interpretive criteria of Ramzi (21) were adopted for recording inhibition zones caused by biocides.

The spore- forming *Bacillus* is ubiquitous in the environment, normally found in soil and vegetation. Both *Bacillus lentus* and *B. subtilis* exhibited resistance to all tested biocides under clean and dirty conditions. Simultaneously, *B. lentus* was resistant to four out of six tested antibiotics and showed heteroresistance, a phenomenon in which a bacterial isolate has small subset of cells that are significantly less susceptible to antibiotics than the main population. Conversely, *B. subtilis* was sensitive to ciprofloxacin and exhibited resistance or heterororesistance to the remaining antibiotics.

In the present study, the four *Bacillus* species tested for their susceptibility to antibiotics and biocides were recovered already from contaminated in-use antiseptics and disinfectants; which may have led to enormous ability to tolerate and adapt to a variety of harsh environmental physical and chemical conditions as they may endure adaptive development and progressively acquire resistance making them as a reservoir for the development of antibiotic cross-resistance (35). For example, chlorination has been associated with a higher incidence of antibiotic resistance (36). Randall et al. (37) have indicated that antibiotic resistant mutant was isolated after treating *S. enterica* with a low concentration of an aldehyde, oxidising, QAC and/or phenolic-based disinfectant agents and after exposing bacteria to an aldehyde-based disinfectant, a mutant resistant to ciprofloxacin was isolated and exhibited either some type of efflux mechanism or a mutation in GyrA. Repeated exposure of E. coli to triclosan has led to decreased susceptibility of *E.coli* (38).
Results reported in the present study

V. Conclusions:

The bacteria under test already exposed to biocides, showed enormous ability to tolerate and adapt to the dirty conditions. They demonstrated reduced susceptibility to biocides and antibiotics which could signify and imply on potential existence of a linkage between biocide usage and antibiotic resistance. This situation therefore, is creating a reservoir for the development of antibiotic cross-resistance.

Ethical Approval:

Approval to conduct the study was obtained from the Ethical committee at Isra University SREC/2 SREC/2 3t031077.

Conflict of interest:

No conflict of interest. This work was part of MSc. thesis of the first author

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