The Efficacy of Combination of Disinfectants or Antiseptics in Overcoming Methicillin-resistant *Staphylococcus aureus*

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This study evaluated the antimicrobial efficacy of five biocides (disinfectants and antiseptics) on multidrug resistant isolates of *Staphylococcus aureus*. Disinfectants and antiseptics, used were chlorohexidine/cetrimide (savlon), povidone-iodine (betadine), ethyl alcohol (ethanol), sodium hypochlorite (chlorax) and glutaraldehyde (cidex). Eighty-three isolates out of 200 collected samples (41.5%) were identified as *S. aureus* and were screened for their sensitivity to methicillin and oxacillin. Two isolates MRSA/ORSA and ORSA were examined for their susceptibility to the above mentioned disinfectants and antiseptics. The highest concentration of these disinfectants and antiseptics were the most effective ones on both isolates. MRSA/ORSA and ORSA became more susceptible after re-inoculation with different kinds of disinfectants and antiseptics except ethyl alcohol which showed no effect. The combination between two disinfectants to overcome the microbial resistance was studied. The inhibition zone of combination between two biocides (chlorhexidine/cetrimide & povidone-iodine) increases a little more than that with each antiseptic alone in case of double resistant isolate. The combination was less inhibitory when chlorhexidine/cetrimide was tested with single resistant isolate, but more inhibitory than the povidone-iodine at both higher and lower concentrations.

Key words: *Staphylococcus aureus*, MRSA, Multidrug resistance, Disinfectants, Antiseptics.

During the last decades, a dramatic increase in hospital-acquired infections caused by multi-drug resistant microbes has taken place. As a result, the threat of microbial contamination and infection has led to the increased use of disinfectants and antiseptics[1]. Antiseptics and disinfectants are used extensively in homes, hospitals and other health care centers to control the growth of microbes on both living tissues and inanimate objects. They are essential parts of infection control practices and aid in the prevention of nosocomial infections[2]. The widespread use of antiseptic and disinfectant products (biocides), as with more-frequent use of antibiotics, contributes to the emergence and/or selection of pathogens that are less susceptible not only to biocides but also to antibiotics[3]. The frequency and spectrum of antibiotic resistant infections have increased, this increase has been attributed to a combination of microbial characteristics, the selective pressure of antimicrobial use, social and technical changes that enhance the transmission of resistant organisms factors (such as increased use and misuse of antimicrobial agents), increased use of invasive devices and procedures, a greater number of susceptible hosts, and lapses in infection control practices[4].

Staphylococcus aureus is the causal agent of most of staphylococcal diseases and is

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currently a versatile microbial pathogen that has evolved resistance to all antibiotic classes. It is associated with serious community-acquired and nosocomial infections[5]. Its high level of adaptation to hospital environments has been deeply facilitated by the acquisition of methicillin resistance, an evolutionary step that converted *S. aureus* to methicillin-resistant *S. aureus* (MRSA), one of the most common nosocomial pathogens nowadays[6]. MRSA is one of the most problematic clinically relevant pathogen and ranks as one of the most difficult bacteria to treat in patients and eradicate in a hospital environment[7].

Relatively recently research has been focusing on the possible development of anti-disinfectant resistance in bacteria, analogous to antibiotic resistance, which has led to complicated treatment and to increased morbidity and mortality[8]. A common problem is the selection of disinfectants and antiseptics because different pathogens vary in their response to different antiseptics and disinfectants[9]. This study was carried out to isolate *S. aureus* strains resistant to antibiotics (MRSA/ORSA) and evaluate the efficacy of biocide agents used in public hospitals, to overcome the resistance of these strains of the microbe.

MATERIAL AND METHODS

Clinical specimens

Swab samples were collected on mannitol salt broth medium, from different departments and indoor environments of three hospitals in Cairo, Egypt. From departments of Intensive Care of Neurosurgery, Chest and Cardiac Surgery and Cardiovascular in Al-Hussein University Hospital, Orthopedic department in Sayed Galall-University Hospital and the medical laboratory of Dar El-Shefa Hospital. Two hundred samples were isolated from different sources of indoor environment within hospitals.

Phenotypic identification of *Staphylococcus aureus*

Pure colonies of Gram positive, cluster-forming, catalase positive staphylococci were used. Isolates were incubated at 37°C for 24 hours on blood agar and then sub-cultured on Tryptic Soya agar. Single colonies were subjected to tube coagulase test and growth on Mannitol salt agar (MSA). To confirm fermentation of mannitol, growth of yellow colonies on MSA (Oxoid, Cambridge, UK) surrounded by yellow zones after 24 hours of incubation at 37°C indicated a positive result. For tube coagulase tests, colonies of test isolates were emulsified in diluted rabbit plasma (bioMérieux, France) in a tube. The tube was kept at 37 ºC and observed for clot after 1 to 4 hours or, if negative, next day[10]. Only eighty-three isolates (41.5%) out of the 200 were subjected to API Staph system (bioMérieux, France) and identified as *S. aureus*.

Antimicrobial susceptibility testing

S. aureus isolates were subjected to phenotypic and genotypic susceptibility testing. The phenotypic method included disc diffusion test. The genotypic method is done for the detection of *mecA* gene. The antibiotic susceptibility of the isolates was examined using the disk diffusion method on Mueller–Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute guidelines[11]. The following antimicrobial disks were tested; penicillin group: penicillin (P-10 ìg), oxacillin (OX-1 ìg), methicillin (ME-5 ìg), amoxicillin/clavulanic acid (AMC-30 ìg), ampicillin/sulbactam (SAM-20 ìg); cephalosporins group: cefotaxime (CTX-30 ìg). Antibiotic discs were placed on MHA plates inoculated with 0.5 McFarland Standardized overnight cultures (containing 10^7–10^8 cfu/ml); plates were then incubated at 35ºC for 18–24 h. Experiments were done in triplicates and the inhibition zone diameter (IZD) was measured. Methicillin resistance was confirmed by the detection of *mecA* gene by PCR[12].

Screening of different concentrations of disinfectants and antiseptics

The two highly resistant isolates of *S. aureus*, [oxacillin resistant (ORSA) and oxacillin and methicillin resistant (ORSA/MRSA)] were tested for their susceptibility to five of the current disinfectants and antiseptics (listed in table 1). Different concentrations were prepared using sterile distilled water added in different volumes to the original preparations solutions of the disinfectants and antiseptics.

Screening for the disinfectant effect was done by agar well diffusion method, inoculating the disinfectant into a MHA plate inoculated with 0.5 McFarland Standardized overnight cultures . Wells of 5 mm diameter were punched in the agar plates and filled with 20 ìl of each concentration of the tested disinfectant and antiseptic. The plates were then incubated at 37°C for 24 h and observed for the zones of inhibition around the wells to identify the suitable concentration that affects the *S. aureus* resistant isolates.

Testing for changes in susceptibility

The growth around the inhibition zone (using the higher and lower concentrations of the disinfectants and antiseptics) for both resistant isolates of *S. aureus* was again inoculated into another MHA plate. The same higher and lower concentrations of the tested disinfectant and antiseptics were inoculated to the middle of the plate and then incubated at 37°C for 24 hours and the IZD was measured to show the effect of the second run on the organism.

Effect of the combination between antiseptics/or disinfectants

The combination of "povidone-iodine/ chlorohexidine" at both higher and lower concentrations was inoculated into the middle of two MHA plates (one for each concentration) with each resistant *S. aureus* strain to show their possible synergistic or antagonistic effect. Plates were incubated at 37°C for 24 hours and the IZD were measured.

Statistical analysis

The recorded data were treated statistically using the one way analysis of variance as described by Snedecor and Cochran[13]. The means were compared by least significant difference (LSD) using SPSS program version 8. The results were considered significant when the difference between the two compared data was higher than the LSD value.

RESULTS

From the 200 bacterial isolates tested only 83 isolates (41.5%) were confirmed as *S. aureus* in the Gram differentiation experiments, the biochemical tests and in API20 used. Strains were screened for their sensitivity to methicillin and oxacillin. A total of 25 (30.1%) isolates were resistant to both oxacillin and methicillin (double resistance MRSA/ORSA), while 26 (31.3%) isolates were found to be only resistant to oxacillin (single resistance, ORSA) and 32 (38.6%) isolates were susceptible to methicillin and oxacillin. Two isolates were selected which are resistant to most antibiotics used. The two isolates; SA1 which is single resistant to oxacillin (ORSA) and SA2 which is double resistant, to methicillin and oxacillin (MRSA/ORSA) were selected to study their susceptibility to five disinfectants and antiseptics currently used in hospitals Table (2).

Generally, the data recorded in table (2) indicated that Chlorohexidine/cetrimide (Savlon(has the highest significant effect on the single and double resistant *S. aureus* than disinfection/ antisepsis effects of the other biocides. In addition, the highest concentrations of all groups of disinfectants and antiseptics used were significantly most effective on both isolates than the lowest concentrations. The statistical analysis revealed that most concentrations within each group have significant difference effect on the single and double resistant *S. aureus*. The disinfection and antisepsis of the five biocides, according to their effect on the single and double resistant *S. aureus*, are divided into two groups*:* the first one (ethyl alcohol) has no effect on the two strains. The second effective group is divided to two subgroups the first one represents the highest effect compound (betadine), while the compounds of the 2nd subgroup have approximately similar effect on the organisms (Fig. 1).

Experiments carried out showed that the isolate with single resistance (SA1) was more resistant to sodium hypochlorite, than that with double resistance. Statistical analysis of the data provided significant differences between both isolates at all concentrations. In both isolates significant differences were detected between the highest concentration and the next lower ones. The higher and lower concentrations of glutaraldehyde(2 & 0.5%) were more effective on the double resistant isolate but the other concentrations (1 &1.5%) were more effective on the single resistant isolate. Statistical significant differences have been found between the effect of glutaraldehyde on either SA2 or SA1 at the different concentrations used. Significant differences were also observed in the single and double resistant isolates between the highest concentrations and the lower ones except with 1.5% in the case of the single resistant isolate. The different concentrations of ethyl alcohol, did not affect both isolates as shown in Table (2).

MRSA/ORSA and ORSA isolates became more susceptible after re-inoculation with different kinds of disinfectant and antiseptic except in case of low concentration of sodium hypochlorite with SA2 and glutaraldehyde with SA1 and SA2 (Table 3).. The re-inoculation with the same disinfectant had a successful effect in the eradication of MRSA/ORSA

An increase in diameter was shown in the inhibition zone of combination between two disinfectants or antiseptics more than with each antiseptic or disinfectant alone in case of double resistant isolate (SA2). The combination between povidone-iodine and chlorohexidine/cetrimide at high concentrations provides a bigger inhibition zone only on the MRSA while ORSA isolate

Table 1. The different concentrations of disinfectants and antiseptics used

Disinfection/ antisepsis	Commercial name	Original conc.	Dilutions
Chlorohexidine(Ch)/cetrimide (c)	Savlon	0.3% (Ch),3% (c)	0.2, 0.1,0.01 %
Povidone-iodine	Betadine	10%	9, 8, 7, 6, 5, 4, 3, 2, 1 %
Ethyl Alcohol	Ethanol	Absolute	90,80,70,60,50%
Sodium hypochlorite	Chlorax	5.25%	1.25, 0.25%
Glutaraldehyde	Cidex	2%	1.5, 1.0 ,0.5%

Table 2. Disinfection and antisepsis effects using different concentrations of the five biocides against single and double resistant *S. aureus*

Zone of inhibition (mm)

Chlorohexidine/cetrimide (Savlon) concentration %

	0.3	0.2	0.1	0.01	LSD (0.05)
SA1	3.35	3.05	2.90	1.95	0.25
SA2	3.15	3.00	2.75	2.35	0.14
LSD (0.05)	0.08	NS	0.05	0.13	-

Glutaraldehyde (cidex) concentration %

	2.0	1.5	1.5	0.5	LSD (0.05)
SA1	2.00	1.95	1.80	1.20	0.07
SA2	2.10	1.80	1.45	1.60	0.17
LSD (0.05)	0.00	0.02	0.03	0.03	-

Ethyl alcohol (ethanol) concentration %

	100	90	80	70	60	50	LSD (0.05)
SA1	0.0	0.0	0.0	0.0	0.0	0.0	-
SA2	0.0	0.0	0.0	0.0	0.0	0.0	-
LSD (0.05)	-	-	-	-	-	-	-

Sodium hypochlorite (chlorax) concentration %

	1.25	0.25	LSD (0.05)
SA1	1.25	1.05	0.18
SA2	1.50	1.45	NS
LSD (0.05)	0.03	0.05	-

Povidone-Iodine (Betadine) concentration

	10%	9%	8%	7%	6%	5%	4%	3%	2%	1%	LSD (0.05)
SA1	1.85	1.60	1.45	1.35	1.25	1.25	1.20	1.25	1.15	0.95	0.07
SA2	2.05	2.00	1.65	1.50	1.50	1.45	1.40	1.35	1.25	0.50	0.06
LSD (0.05)	0.03	0.06	0.05	0.05	0.03	0.06	0.03	0.11	0.12	0.23	-

(M/O): Methicillian & Oxacillin
(O): Oxacillin

Table 3. Changes in susceptibility of *S. aureus* after re-inoculation with higher and lower concentrations of Chlorhexidine, Povidone-iodine, sodium hypochlorite and Glutaraldehyde

Concentration	M/O resistant *S. aureus* (IZD mm)			O resistant *S. aureus* (IZD mm)		
	1st inoculation	2nd inoculation	LSD (0.05)	1st inoculation	2nd inoculation	LSD (0.05)
Chlorohexidine/cetrimide						
0.3%	3.15	5.25	0.30	3.35	5.60	0.24
0.01%	2.35	2.45	0.41	1.95	3.75	0.11
LSD (0.05)	0.27	0.35	-	0.04	0.62	-
Povidone-iodine						
10%	2.05	4.85	0.52	1.85	5.75	0.91
1%	0.50	4.25	0.36	0.95	4.25	1.10
LSD (0.05)	0.15	0.79	-	0.04	1.85	-
Sodium hypochlorite						
5.25%	3.05	5.25	0.26	4.30	6.75	0.53
0.25%	1.45	0.0	0.02	1.05	3.70	0.34
LSD (0.05)	0.06	0.04	-	0.07	0.12	-
Glutaraldehyde						
2%	2.10	2.45	0.03	2.00	2.40	0.08
0.5%	1.60	0.0	0.03	1.20	0.0	0.00
LSD (0.05)	0.04	0.02	-	0.0	0.03	-

(M/O): Methicillian & Oxacillin (O): Oxacillin

Table 4. Effect of the combination between the two antiseptics (Povidone-iodine and Chlorhexidine/cetrimide) on the two strains of *S. aureus*

	M/O resistant *S.aureus* (IZD mm)				O resistant *S.aureus* (IZD mm)				Combination	
	Povidone - iodine	Chlorhexidine/ cetrimide	Combination of both	LSD (0.05)	Povidone- iodine	Chlorhexidine / cetrimide	Combination of both	LSD (0.05)	LSD (0.05)	LSD (0.05)
Higher conc	2.05	3.15	3.50	0.06	1.85	3.35	3.10	0.12	0.25	
Lower conc	0.50	2.35	2.40	0.08	0.95	1.95	1.85	0.16	0.40	
LSD (0.05)	0.08	0.05	0.09	-	0.03	0.02	0.04	- -		

(M/O): Methicillian & Oxacillin (O): Oxacillin

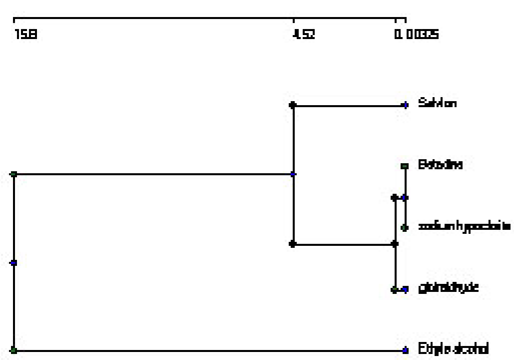

Fig. 1. Clustering analysis technique of five disinfectants and antiseptics according to their effect on the single and double resistant *S. aureus*

showed higher resistance with an inhibition zone which was found decreased than that of chlorhexidine/cetrimide alone (Table 4). No significant difference was found between the effect of the combination on single and double resistant isolates at both high and low concentrations. The combination was less effective on single resistant strain than the effect of chlorhexidine/cetrimide alone, but more effective than the povidone-iodine alone at both higher and lower concentrations.

DISCUSSION

Different types of microorganisms vary in their responses to antiseptics and disinfectants because of their different cellular structures, composition and physiology. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of those microorganisms of increasing concern in the realm of healthcare. MRSA is a strain of *Staphylococcus aureus* that is resistant to beta-lactam antibiotics[14]. It is a multidrug–resistant *S. aureus* which is responsible for many human infections that are difficult to treat (Klein *et al*., 2007) hence, it is a major cause of both nosocomial and community-acquired infections[14,15].

Staphylococcus aureus may have acquired resistance to some antiseptics and disinfectants as mentioned by Russell[16]. It has been known for several years that some antiseptics and disinfectants are somewhat less inhibitory to *S. aureus* strains that contain a plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamicin[17,18].

Saha et al[18] suggest that the antibacterial effects of antiseptics and disinfectants are not only dependent on the types of antiseptics and disinfectants but also on their concentrations. The nature of action of disinfectants was very encouraging as it revealed activities at varying degrees with different concentrations against the bacterial isolates indicating that they can eradicate MRSA from the environment. The goal of disinfection is to reduce the risk of endemic and epidemic nosocomial infections in patients. A great number of disinfectants are used in the healthcare settings which are sporicidal chemicals when used in appropriate concentrations and are recommended for care of patients, items and instruments[19].

It has become clear that some antiseptics and disinfectants on the one hand and antibiotics on the other have similar effects on bacteria[20]. Murtough et al.[21] found that, rotation of disinfectants in hospitals and elsewhere, e.g. in the pharmaceutical and food industries, has been advocated to prevent the development of bacterial resistance. It has been claimed that, ideally, one disinfectant should be replaced by another having a dissimilar mechanism of action. Due to the high resistance of bacteria to antibiotics, the use of disinfectants with a broad spectrum of action is of great importance, since the elimination of these bacteria would prevent further spreading.

Comparing the antimicrobial efficacy of antiseptics and disinfectants used in this study, chlorhexidine/cetrimide (Savlon) showed better antibacterial efficacy against MRSA than other biocides used in this study. Such result is explained by McDonnell and Russell[1] who illustrated that high concentration of chlorhexidine (CHX) causes coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage, so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of CHX increases, but leakage is reduced at higher concentrations because of the coagulation of the cytosol. This contrasts with Köljalg et al.[22] who found that CHX acts through the damage of the outer cell layers and crosses the cell wall or outer membrane, then attacks the bacterial cytoplasm or inner membrane causing leakage of intracellular constituents[23].

Povidone-iodine also affected both single and double resistant *S. aureus* but in a rate lower than CHX. According to Mycock[24], MRSA strains show a remarkable increase in tolerance (at least 5,000-fold) to iodine. The different concentrations of ethyl alcohol show no effect on the succeptibility of single or double resistant *S. aureus*. This result can be explained in the light of the work of Klein and Deforest[25] who stated that isopropyl alcohol is considered slightly more efficacious against bacteria than ethyl alcohol which is more potent against viruses. An opposite result was found by Suzuki et al.[26] who tested 70% alcohol against *S. aureus* methicilin susceptible and resistant (MRSA), they indicated that no viable counts were found after 1 to 3 minutes. Although it is almost universally recognized as an effective agent, Ethanol use is fraught with controversy and conflicting findings[27]. Campos et al.[28] found that, Ethanol is not effective against all isolates, and 64.2% of *S. aureus* strains are resistant to this disinfectant. This result may have occurred due to volatilization so that Ethanol requires time to act efficiently on bacterial proteins denaturation.

Our results indicate that, the most effective concentration of glutaraldehyde is 2%. It could affect both ORSA and ORSA/MRSA isolates and as the concentration decreases its effect decreases. Cardoso et al.[29], reported that 2% glutaraldehyde solution is bactericidal in 1 min. and sporicidal in 15 min. Vieira et al.[30] recommend glutaraldehyde 2% concentration as a sterilizing solution and high-level disinfectant. Previous studies demonstrate a strong binding of glutaraldehyde to outer layers of organisms such as *Staphylococcus aureus* [31].

The re-inoculation with disinfectant and antiseptics reveales a difference in the inhibition zone diameter. The highly effective disinfectant in the first cycle is also the highly effective disinfectant in the second cycle. The increase effective in almost all cases except at low concentrations of sodium hypochlorite with SA2 and glutaraldehyde with SA1 and SA2, this means that the organism grown around the clear zone in the first cycle was weakened and became more susceptible. Maraha et al.[32], found after two treatment cycles, that the rate was 91.4% and came up to 94.2% after a third treatment cycle. This result contrasts with results obtained by McDonnell and Russell,[1] who found that *S. aureus* as mucoid strains, with the cells surrounded by a slime layer which plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules. The role of combination of two disinfectants or antiseptics in enhancing or antagonizing their effect on *S. aureus* (MRSA or ORSA) is still unclear. Kampf and Kramer[33] stated that eradication of MRSA/ORSA from colonized patients is regarded as a key element in prevention of transmission in a hospital.

CONCLUSIONS

The increased emergence of antibiotic resistant bacteria suggests the need for heavier reliance on disinfection practices to prevent initial infection. The goal of antisepsis and disinfection is to reduce the risk of endemic and epidemic nosocomial infections in patients. The results of this study suggested that alternatives to infection control can be considered, such as combination between two disinfectants or antiseptics, proving their effectiveness in disinfection. The findings of this study should assist in reducing the occurrence of nosocomial infections and, therefore, the morbidity, mortality and socio-economic burden caused by prolonged hospitalization.

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