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Full Length Research Paper

The bactericidal and fungicidal effects of salicid on pathogenic organisms involved in hospital infections

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The study was designed to investigate bactericidal and fungicidal actions of salicid (pH: 6.7, ORP: 760 mV, residual chlorine of 2 to 5 ppm) on hospital infections. Four of the most common opportunistic pathogens (*Klebsiella pneumoniae*, *Staphylococcus aureus*, *Rhizopus oryzae*, and *Aspergillus fumigatus*) were used for this study. Cultures were inoculated in 9 mL of salicid and incubated for 0.5, 1, 3, 5, 7 and 10 min at room temperature (23 ± 2°C). A dipping method was followed for this study. Untreated pathogens were treated as control. Compared to the untreated control, a reduction of 1.10 to 6.08 $\log_{10}$ CFU/mL for aforementioned pathogens were recorded as the result of treatment with 2 and 5 ppm salicid, respectively. The highest bactericidal effect was found with *S. aureus* for 5 ppm salicid with 0.5 min immerse time. Salicid treatment with 0.5 min dipping and 5 ppm residual chlorine also reduced *K. pneumoniae*, *R. oryzae*, and *A. fumigatus* by 5.00, 3.50 and 2.63 $\log_{10}$ CFU/mL, respectively. Our findings showed that in each pathogen, efficacy of salicid decreased significantly ($p < 0.05$) with increased dipping time, from 0.5 to 10 min and there was significant difference ($p < 0.05$) observed between 2 and 5 ppm salicid treatment in reducing pathogens. The results indicate that salicid may be a useful disinfectant for hospital infections, but its clinical application has still to be evaluated.

Key words: Salicid, bactericidal and fungicidal effect, hospital infections.

INTRODUCTION

Hospital infections are a serious medical, social, and economic problem for public health services all over the world (Vorobjeva et al., 2004). *K. pneumoniae*, *S. aureus*, *R. oryzae*, and *A. fumigatus* are the most opportunistic human pathogens involved in hospital infections. *K. pneumoniae* is a major cause of nosocomial infections (DeChamps et al., 1991; Johnson et al., 1992). As a general rule, Klebsiella infections tend to occur in people with a weakened immune system. Many of these infections are obtained when a person is in the hospital for some other reason. The most common infection caused by *Klebsiella* bacteria outside the hospital is pneumonia. *S. aureus* is the most common organism responsible for postoperative wound infections (Wenzel and Perl, 1995) and a leading cause of septicaemia, intravenous catheter-related infections and skin and soft-tissue infections. Auto-infection of surgical wounds by *S. aureus* is common, and is associated with considerable morbidity and represents an important medical and economic problem. *R. oryzae* is the most common cause of zygomycosis, a life-threatening infection that usually occurs in immunocompromised patients (Ibrahim et al., 2005). Infections caused by *Aspergillus* species have grown in importance in recent years (Pasqualotto, 2009). As most of the *Aspergillus* infections are caused by *A. fumigatus*, the majority of studies have focused on this species. Such pathogens are typically characterized by a wide variety of sources, ways, and factors of transmission, appearing in different types of clinics and preventive hospitals. In this context, the methods of asepsis and active chemical antisepsis are currently becoming increasingly important in terms of the prevention of hospital infections.

Salicid is the first potable product treating infectious disease on human mucosa and skin using its

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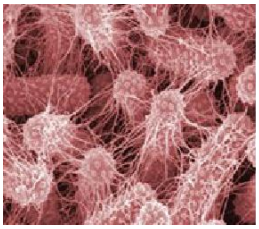
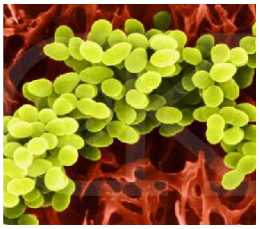
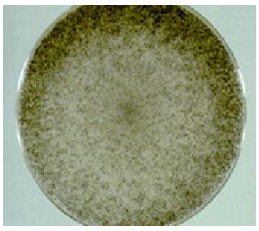


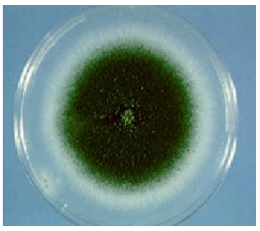
Klebsiella pneumoniae



Staphylococcus aureus



Rhizopus oryzae



A. fumigatus

Figure 1. Challenge microorganism.

antimicrobial efficacy for bacteria, fungi and viruses. When electrolyzing brine with salicid, small sized chlorine bubbles are generated and this chlorine gas dissolves in the solution, producing the free chlorine. Salicid hardly change the pH value of used water, so it is the product available for medical use which can use hypochlorous acid which is a predominant species of free chlorine at the pH range of weak acidic and neutral. The free chlorine solution generated by salicid is below 5 ppm which is allowed by WHO for drinking water and swimming pool water and also is safe to human mucosa. Accordingly, salicid is the first antiviral product which can be applied for human mucosa and is the first product which has antimicrobial efficacy for bacteria and fungi, simultaneously.

The objective of this study was to evaluate the bactericidal and fungicidal effect of salicid obtained in the salicid electrolysis device on common hospital pathogens under *in vitro* conditions.

MATERIALS AND METHODS

Challenge microorganisms

K. pneumoniae- ATCC 8724, *S. aureus*- ATCC 12488, *R. oryzae*- ATCC 24794, *A. fumigatus*- ATCC 26430 (Figure 1).

Media and reagents

Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), Potato Dextrose Agar (PDA), 0.85% saline solution (ss), Neutralizer used: 0.85% Nacl containing 0.5% $Na_2S_2O_3$

Laboratory equipments

Sterile test tubes, Sterile flasks, Sterile Beaker, Sterile pipettes, Petridishes, Stopwatch, Incubator capable of maintaining 35°C, Incubator capable of maintaining 25°C, Pincer, Cheese gauge cloth, Haemocytometer, Microscope, Glass rod, Calibrated thermometers

Inocula preparation

K. pneumoniae and *S. aureus* stock cultures were transferred into tryptic soy broth (TSB) and incubated for 24 h at 35°C. Following incubation, 10 ml of each culture was sedimented by centrifugation (3000 x g for 10 min), washed and resuspended in 10 ml of 0.1% peptone water (pH 7.1) to obtain a final cell concentration of 10^9 CFU/mL. The bacterial population in each culture was confirmed by plating 0.1 ml portions of appropriately diluted culture on tryptic soy agar (TSA) (Difco Laboratories) plates and incubating the plates at 35°C for 24 h. 1 ml of the suspension was used as the inoculum (10^9 CFU).

The two fungal strains *A. fumigatus* and *R. oryzae* were inoculated from the stock culture onto PDA and incubated at 25°C for 10-15 days or until sporulation occurred. When the cultures appeared to be mature, the mycelial mats was removed from the surface of at least five plates and macerated with ss with a sterile glass rod. The suspension was filtered through sterile cheese gauge cloth to remove the hyphae. In order to remove any organic matter, each of the prepared fungi suspensions was washed in the same manner as described for the bacteria.

Test agent preparation

For each generation of the test material, one salicid packet was added to the test device containing 35 mL of sterile tap water (pH 7.0). The device was shaken 10 times. The generation button was activated by pressing the button and let it electrolysis for 20 s. After 20 s the activation button was stopped manually, then the device was shaken 2-3 times and the product was immediately dispensed for testing. After 20 to 21 s electrolysis we got 2 ppm salicid and in the same way after 44-45 s electrolysis we got 5 ppm salicid (pH: 6.5-7.0, ORP: 760-775 mV).

Test devices and material

Salicid (device), serial no. 001170, Salicid (device), serial no. 001171, Salicid salt packet, (Executive Summary former salt 315 mg/ saline 35 ml), DPD Free Chlorine Reagent (Figure 2).

Test

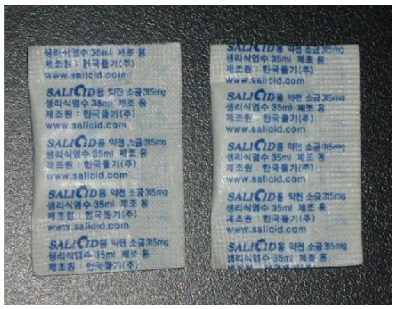
For each replicate, a 1 mL aliquot of prepared inoculum was added to a sterile test tube. Once the test material is generated, 9 mL of the prepared test agent was added to the tube containing 1 mL of the prepared inoculum; a timer started and the tube was mixed immediately. After each contact times, a 1 mL sample was transferred to a tube containing 9 mL of neutralizer. Serial ten-fold dilutions were performed in 0.85% ss dilution blanks. There were three replicates performed for each microorganism.

Contact times

30 s, 1 , 3, 5, 7 and 10 min.

Salicid device

Dry cell



Salicid salt packet

Figure 2. Test devices.

Temperature

Ambient room temperature

Incubation and enumeration

Upon completion of the test, all plates were inverted and incubated for the appropriate time and temperature as follows: *k. pneumoniae* and *s. aureus* were incubated for 18 to 24 h at 35°C. *A. fumigatus* and *R. oryzae* were incubated for 3-5 days at 25°C. Following incubation, all plates were removed from the incubator, the colonies were counted and the CFU/mL at each contact time was determined.

Statistical analysis

For each treatment, the data from the independent replicate trials were pooled and the mean value and standard deviation were determined.

RESULTS AND DISCUSSION

The mean counts, percent reduction and $\log_{10}$ reduction per test material, contact time and organism are presented in Tables 1 to 4. $\log_{10}$ reduction and percent reduction were calculated using the following equations:

Enumeration is expressed as colony-forming units (CFU/mL)

$$\frac{\text{Average Initial Counts Control} - \text{Test Results}}{\text{Average Initial Counts Control}} \times 100 = \text{Percent Reduction}$$

$\log_{10}$ (Average Initial Counts Control) – $\log_{10}$ (Test Results) = $\log_{10}$ Reduction

Cell suspensions of *K. pneumoniae*, *S. aureus*, *R. oryzae* and *A. fumigatus* were treated at RT (23 ± 2°C) for 0.5, 1, 3, 5, 7 and 10 min with Salicid. Salicid had major antibacterial activity compared to yeast and mould on different challenge microorganisms. Our results revealed that salicid containing 5 ppm of residual chlorine was more effective ($p < 0.05$) than that of 2 ppm salicid in reducing populations of bacterial and fungal strains regardless of dipping time. Reduction of bacterial count was ranged from 3.15 to 6.08 $\log_{10}$ CFU/mL and 1.10 to 3.50 $\log_{10}$ CFU/mL reduction was gained in yeast and mould, respectively. With the increased dipping time rate of log reduction was decreased. The available chlorine concentration (ACC) reduced with an increase in dipping time which could have resulted in lower reductions at increased dipping times. Sensitivity to sanitizers depends on pathogens' characteristics and properties of sanitizers. From our tested pathogens bacteria were more sensitive to salicid compared to fungi and *A. fumigatus* was more resistant to salicid treatment than other three pathogens. Chemical compounds such as formaldehyde, phenol, glutaraldehyde, tricresol and chloramines have shown bactericidal and fungicidal effects (Hegna and Clausen, 1988). However, most of these disinfectants are made from the dilution of condensed solutions, which in handling involves some risk and is troublesome. A disinfectant that is not produced from the dilution of a hazardous condensed solution is required for practical use.

In recent years, electrolyzed oxidizing water (EOW) has gained interest as a disinfectant used in agriculture, dentistry, medicine and food industry (Huang et al., 2008). An advantage of EOW is that it can be produced with tap water, with no added chemicals other than sodium chloride. EOW has been attracting as a disinfectant because of its strong microbicidal activities on a broad variety of bacterial pathogens (Venkitanarayanan et al., 1999). Moreover, EOW exerts fungicidal activity (Suzuki et al., 2002a, b). Xiong et al. (2010) reported that the OH radical ($^{\cdot}OH$) that exists in neutralized electrolyzed oxidizing water (NEW) and acidic electrolyzed oxidizing water (AcEW) was found to have an important fungicidal factor. However, the potential application of EOW is limited because of its low pH values (≤ 2.7) and it corrosive characteristics. At this low pH, dissolved Cl_2

Table 1. *In vitro* inactivation of *Klebsiella pneumoniae* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
1.0×10^8	0.5	$1.5 \times 10^4 \pm 0.10$	$1.0 \times 10^3 \pm 0.06$	99.9850	99.9990	3.82^a	5.00^b
	1	$1.6 \times 10^4 \pm 0.09$	$1.0 \times 10^3 \pm 0.09$	99.9840	99.9990	3.80^a	5.00^b
	3	$1.9 \times 10^4 \pm 0.10$	$1.5 \times 10^3 \pm 0.20$	99.9810	99.9985	3.72^a	4.82^b
	5	$2.8 \times 10^4 \pm 0.18$	$2.5 \times 10^3 \pm 0.09$	99.9720	99.9975	3.55^a	4.60^b
	7	$5.5 \times 10^4 \pm 0.10$	$6.5 \times 10^3 \pm 0.06$	99.9450	99.9935	3.26^a	4.19^b
	10	$7.0 \times 10^4 \pm 0.08$	$1.1 \times 10^4 \pm 0.13$	99.9300	99.9890	3.15^a	3.96^b

Values with different letters within the same column differ significantly at $p < 0.05$.

Table 2. *In vitro* inactivation of *Staphylococcus aureus* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
1.2×10^8	0.5	$3.5 \times 10^2 \pm 0.10$	$1.0 \times 10^2 \pm 0.1$	99.9997	99.9999	5.54^a	6.08^b
	1	$4.0 \times 10^2 \pm 0.10$	$1.0 \times 10^2 \pm 0.26$	99.9997	99.9999	5.48^a	6.08^b
	3	$6.0 \times 10^2 \pm 0.18$	$1.5 \times 10^2 \pm 0.17$	99.9995	99.9999	5.30^a	5.90^b
	5	$1.1 \times 10^3 \pm 0.21$	$2.0 \times 10^2 \pm 0.18$	99.9991	99.9998	5.04^a	5.78^b
	7	$1.5 \times 10^3 \pm 0.20$	$3.0 \times 10^2 \pm 0.25$	99.9988	99.9998	4.90^a	5.60^b
	10	$2.0 \times 10^3 \pm 0.18$	$4.5 \times 10^2 \pm 0.22$	99.9983	99.9996	4.78^a	5.43^b

Values with different letters within the same column differ significantly at $p < 0.05$.

gas can be rapidly lost due to volatilization, adversely affecting human health and the environment. Moreover, the high acidity of EOW may cause the corrosion of equipment and consequently limit its practical application (Abadias et al., 2008; Guentzel et al., 2008). So, as an alternative to EOW we used salicid in our study. Salicid with a pH value of 6.5–7.0, also known as slightly acidic low concentration electrolyzed water (SlALcEW), is commonly produced by electrolyzing a dilute salt solution (0.9% NaCl) in a non-membrane electrolytic cell (Rahman et al., 2010). At a near-neutral pH, the predominant chemical species is the highly biocidal hypochlorous acid species (HOCl, approximately 95%). The advantage of salicid are numerous: non-corrosive due to near-neutral pH, low current and minimum time required to produce it, it does not leave residuals due to low content of ACC (2 - 5 mg/L), comparatively inexpensive, and a less potential health hazard to the worker due to the lack of Cl_2 off-gassing. To produce salicid, an apparatus is required that utilizes common salt and an electric source. Salicid can be produced at site, as the size of the machine is quite small. Therefore, the widely used EOW might be replaced by salicid as an effective and environmentally friendly sanitizer in medical use.

Based on our study this salicid can be recommended for use as a strong disinfectant for the equipment, and diagnostic and medical devices in hospitals. However, future clinical research has to be done under an *in vivo* system, in order to

Table 3. *In vitro* inactivation of *Rhizopus oryzae* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation Units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
7.5×10^6	0.5	$7.4 \times 10^3 \pm 0.13$	$2.4 \times 10^3 \pm 0.09$	99.9013	99.9680	3.01^a	3.50^b
	1	$8.0 \times 10^3 \pm 0.11$	$2.5 \times 10^3 \pm 0.01$	99.8933	99.9667	2.98^a	3.48^b
	3	$1.1 \times 10^4 \pm 0.09$	$3.6 \times 10^3 \pm 0.08$	99.8533	99.9520	2.84^a	3.33^b
	5	$1.5 \times 10^4 \pm 0.08$	$5.3 \times 10^3 \pm 0.04$	99.8000	99.9293	2.70^a	3.16^b
	7	$2.7 \times 10^4 \pm 0.11$	$1.2 \times 10^4 \pm 0.09$	99.6400	99.8400	2.45^a	2.78^b
	10	$4.8 \times 10^4 \pm 0.08$	$3.0 \times 10^4 \pm 0.29$	99.3600	99.6000	2.20^a	2.41^b

Values with different letters within the same column differ significantly at $p < 0.05$.

Table 4. *In vitro* inactivation of *Aspergillus fumigatus* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation Units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
2.4×10^7	0.5	$2.6 \times 10^5 \pm 0.05$	$5.6 \times 10^4 \pm 0.06$	98.9167	99.7667	1.97^a	2.63^b
	1	$3.0 \times 10^5 \pm 0.03$	$6.0 \times 10^4 \pm 0.03$	98.7500	99.7500	1.90^a	2.60^b
	3	$4.0 \times 10^5 \pm 0.26$	$9.0 \times 10^4 \pm 0.05$	98.3333	99.6250	1.78^a	2.43^b
	5	$6.0 \times 10^5 \pm 0.20$	$1.4 \times 10^5 \pm 0.11$	97.5000	99.4167	1.58^a	2.23^b
	7	$1.0 \times 10^6 \pm 0.23$	$1.8 \times 10^5 \pm 0.08$	95.8333	99.2500	1.38^a	2.13^b
	10	$1.9 \times 10^6 \pm 0.10$	$2.5 \times 10^5 \pm 0.04$	92.0833	98.9583	1.10^a	1.98^b

Values with different letters within the same column differ significantly at $p < 0.05$.

evaluate the stability of salicid under operating conditions and its safety for medical personnel and patients.

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