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DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS OF COMMON BIOCIDES TO CLINICALLY SIGNIFICANT *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

Background: Not many studies have explored the susceptibility profile of nosocomial pathogens like *K pneumoniaeto* biocides. Hence, this study aimed at determining the minimum inhibitory concentrations (MIC) of clinically significant *Kpneumoniae* to two commonly used biocides: chlorhexidine (2.5%) and isopropyl alcohol (100%). **Methods:** The in *vitro* susceptibility of 97 strains of *K pneumonia* positive for ESBL production and 83 strains of *K pneumonia* negative for ESBL production to chlorhexidine and isopropyl alcohol was studied. Broth macro dilution according to standards set by CLSI guidelines and timekillassaywasdonetostudythesusceptibilityofclinicallysignificant. *Kpneumoniae* to chlorhexidine and isopropyl alcohol. **Results:** The MIC of Chlorhexidine for *Kpneumoniae positive* for ESBL production was in the range of 0.078 to 0.625mg/ml and *K pneumonia* negative for ESBL production was in the range of 0.078to0.313mg/ml. The MIC of isopropylalcohol for *Kpneumoniae* positive for ESBL production was in the range of 0.975 to 15.6mg/ml and *K Pneumoniae* negative for ESBL production was in the range of 0.975 to 7.8mg/ml. **Conclusion:** *K pneumonia* positive for ESBL production showed reduced susceptibility to chlorhexidine (2.5%) and isopropyl alcohol (100%) when compared to *K pneumonia* negative for ESBL production.Morestudiesarerequiredtovalidateourfindingsofresistancetobiocidesin clinically significant nosocomial pathogens.

INTRODUCTION

Klebsiella belongs to the family Enterobacteriaceae. *Klebsiella spp.* has many characteristics that promote their virulence. *Klebsiella spp*. expresses two types of antigens on their cell surface, capsular antigen and lipopolysaccharide antigen. Both antigens play an important role in classification, Epidemiology and pathogenicity of *Klebsiellaspp*[1]. It has a prominent capsule which enhances the virulence of the organism in-vivo. *K pneumoniae* is the most important *Klebsiella*species from a medical standpoint

and most commonly isolated from clinical specimens. *K pneumoniae* is also associated with nosocomial infections in long term hospitalized patients. *K pneumonia* is known to cause wound, soft tissue infections and urinary tract infections [2, 3] Hospitals routinely use biocides like isopropyl alcohol for infection control. Activity is optimal when used at a concentration of 60-90%. This concentration of isopropyl alcohol is bactericidal but not sporicidal [4].Medical device manufacturers use isopropyl alcoholroutinely in clean rooms and on a variety of surfaces as a disinfectant, 70% ethyl alcohol and 35% isopropyl alcohol are effective skin antiseptics in the absence of organic matter. Human immunodeficiency virus (HIV) is susceptible to the above concentrations of ethyl alcohol and isopropyl alcohol. Isopropyl alcohol is a better fat solvent more bactericidal and less volatile when compared to ethyl alcohol [5].Ethyl or isopropyl alcohol evaporates quickly. Therefore, either is best used as an antiseptic or on thermometers and injection vial rubbersepta as disinfectants [6].Chlorhexidine is a chlorinated phenolthatis widely used as a hand disinfect antpriorto surgery ("surgical scrub"), in wound disinfection and in mouthwashes[7]. These compounds are bactericidal but not sporicidal. Salvon (chlorhexidine&cetrimide) is widely used in wounds, pre-operative disinfection of skin and as bladder irrigant. Chlorhexidine has more activity against tubercle bacilli, spores and fungi. 4%ChIorhexidine is used as hand sanitizer and in pre-surgical asepsis. Chlorhexidinein alcoholic solution prevents cross contamination with *Pseudomonas* and *Proteus*spp.0.5% chlorhexidine is used to clean contact lenses and as an antiseptic. A minimum of 1 min exposure to chlorhexidine is required for effective action [8]. Activity of chlorhexidine against gram negative bacilli has not been well documented. There are concerns about possible emergence of biocide resistance in *K pneumoniae.*[9] The lowest concentration of an antimicrobial agent that inhibits the visible growthof a microorganism after a period of incubation is defined as Minimum Inhibitory Concentration (MIC) of an antimicrobial agent.[10]. Clinical and research laboratories perform MICs for antimicrobial agents to confirm resistance and for determining the efficacy of new antimicrobial agents and their MIC breakpoints.[11]Clinical and Laboratory Standards Institute(CLSI) give clear guidelines and test methodologies that describe susceptibility or resistance profile withMIC break points for antimicrobials.[13,14] There are no clear guide lines that describe susceptibility or resistance profiles withMIC breakpointsforbiocides. The MIC values of biocides against clinical isolates have been analysed by several researchers but these provide a limited data for multi- drug resistant organisms [15].A study by Guoetal; suggests that monitoring the reduced susceptibility of carbapenem resistant *K pneumonia* to common biocides may help in prevention of improper disinfection. The same study revealed that pan resistant *K pneumonia* were relatively resistant to common biocides such as chlorhexidine, iodophorandethyl alcohol [2]. But a study by Vijayakumaretal,on susceptibility pattern of *Kpneumoniae* strains to biocides like benzalkoniumchloride and chlorhexidinedidnot show reduced susceptibility [11]. Higher morbidity and mortality is seen in nosocomial infections caused by *K pneumoniae. K pneumonias* exist in mixed infections and can cause secondary infections with other pathogenic bacteria. Multi drug resistant *Kpneumoniae* can cause community acquired infections and are difficult to treat with the existing antibiotics [16]. Studies have shown that the increased exposure to broad-spectrum antibiotics may lead to multidrug resistance. ESBL production in bacteria was first identified in 1980. Subsequently in 1983 out breaks of infections caused by *Kpneumoniae* producing ESBL were identified in Europe, US, and South America has been described [17]. Important human pathogens are now known to be resistant to multiple antimicrobials, and treating infections caused by such organisms is a challenge while biocides play an important role in limiting infection, there is concern about the development of resistance in microorganisms to biocides [9].The possible emergence of resistance to biocide among the sestra in sposesagenuine threat of disrupting ouron-going efforts for implementing appropriate and effective measures to control infection. To monitor the biocide resistance rates of such strains should be carried out on a regular basis so that appropriate steps can be

taken before hand to combat the possibility of emergence of a dreaded pandemic [16]

Materials and Methods:

It was a prospective, cross sectional time bound study, the present study was conducted in the Department of Microbiology, Kasturba Medical College (KMC), Mangalore. The study included isolates of *K pneumoniae*from various clinical specimens received at Department of Microbiology, KMC, Mangalore and Microbiology Diagnostic Centre, KMC hospital Ambedkar Circle, Mangalore. The study period was from 01-Nov-2018 to31-April-2018.The study was conducted after obtaining clearance from the Institutional Ethics committee, KMC, Mangalore. The study included clinically significant *Kpneumoniae* which are positive and negative for ESBL production. Strains of *Kpneumoniae* isolated along with other organisms were not included in the study. Isolates of *K pneumonias* from clinical samples without inflammatory cells were excluded from the study. *Kpneumoniae*isolates(n=180)fromclinicalsamples likeBlood,Urine,Swabs,Pusand Aspirates were included in the study. *K pneumoniae*ATCC 700603 (ESBL producer) and *K pneumoniae*ATCC 13883 (Non ESBL producer) were used as control strains. Biocides chlorhexidinegluconate (2.5%) & isopropyl alcohol (100%) were used to study the susceptibility of the *K pneumoniae*strains. All media and chemicals were procured from Hi Media Laboratories Pvt Ltd. Mumbai, India.

Inoculum Preparation: Isolated *Kpneumoniae* was sub-cultured on blood agar and incubated overnight at 37°C. From the incubated blood agar plate a single colony was taken and suspended in Mueller Hinton broth (MHB), and incubated for 4-6 hours at 37°C. The turbidity of MHB was adjusted to McFarland 0.5 standard containing approximately 1.5×10^8CFU/ml. Final inoculum was prepared by diluting 1ml of inoculum and 9 ml of fresh MHB (1:10)[12]

Minimum Inhibitory Concentration by Broth Dilution Method: 2ml of MHB was pipetted into ten sterile test tubes. 2ml of disinfectant was added to the first test tube containing 2ml of MHB and mixed well. From the first test tube serial dilutions was performed till ninth test tube and 2ml of the mixture was discarded from ninth tube. The tenth tube was used as a broth control. 0.01ml of bacterial inoculum was added to each test tube including the control tube. The inoculated tubes were incubated over night at 37°C.

The growth controls *K pneumoniae*ATCC 700603 &*K pneumonia* ATCC 13883 was included in each test. Highest dilution tube showing no turbidity was considered as MIC.

Minimum Bactericidal Concentration: Subculture on nutrient agar was done from all the cleartubes. Highest dilution of biocide not showing any growth on nutrient agar was considered as MBC.[12,13,14]

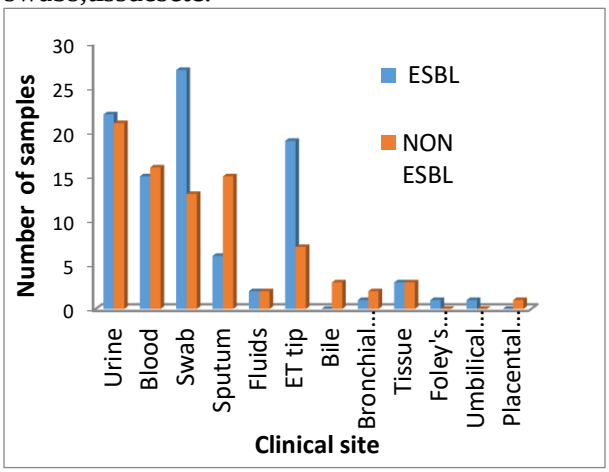
Time Kill Assay: The killkinetics of biocides was determined by time kill assay. *Kpneumoniae* was grown on blood agar plate for 24 hours at 37°C. A single colony was inoculated into MHB and incubated for 6 hours at37°C.

The prepared inoculum was adjusted with McFarland 0.5 standard. Three test tubes labelled as undiluted, 1:2 dilution and saline control were used. To the undiluted test tube 2ml ofbiocide,tothe1:2dilutiontesttube 1ml ofbiocide& 1ml of MHB was added andtothesalinecontrol2ml ofsalinewasadded. 20µl ofbacterialinoculumwasadded to each test tube and incubated at 37°C. 0.01µl from each tube was spread on nutrient agarat0,2,4hours.Theplateswereincubated at37°Cfor48handviable was determined [15, 16].

Data Analysis: p- value was calculated by student t-test using SPSS v.25 statistical analysissoftware (IBM Corporation New York, USA). For comparing the susceptibility to biocides such as chlorhexidine (2.5%) and isopropyl alcohol (100%) and Time kill assay, Pearson's chi-square test wasused.

Results: During the study period 180 clinical isolates, 97 ESBL positive and 83 ESBL negative *K pneumonia* were selected after analysing their antibiogram. As shown in figure 1, isolates were from different sites which include urine, blood, aspirates, sputum, pus,

swabs,tissuesetc.



(Figure 1:Distribution of *K pneumoniae* from different clinical sites)

The MIC of two biocides (100% isopropanol and 2.5% chlorhexidine gluconate) for 97 ESBL and 83 Non-ESBL producing strains of *Kpneumoniae* were determined. The MICs of chlorhexidine and isopropyl alcohol by broth dilution technique is shown in. Table.1&2.The MIC range of chlorhexidine for 97ESBL producing *Kpneumoniae*was seen between 0.078 to 0.625mg/ml and for 83 Non ESBL producing *K pneumonia* it was between 0.078 to 0.313mg/ml. The MIC range of isopropyl alcohol for 97 ESBL producing*Kpneumoniae* was seen between 0.975to15.6mg/ml and for 83 Non ESBL producing *K pneumoniae*it was between 0.975 to7.8mg/ml.

MBC: The MBC of chlorhexidine for 97 (37%) strains of *K pneumoniae*positive for ESBL production was seen at 0.156mg/ml and for 83 (45%) strains of *Kpneumoniae*negative for ESBL production was seen at 0.078mg/ml. The MBC of isopropyl alcohol for 97 (42%) ESBL positive *K pneumonia* was seen at 3.9mg/ml and for 83 (46%) ESBL negative *K pneumoniae*it was at 1.95mg/ml.

Statistical analysis of MIC and MBC:

The possible correlation between the MIC of *K pneumonia* which are positive and negative for ESBL production was statistically evaluated. A significant positive association ($p<0.001$)betweenESBLproductionandMICforeachbiocidewasfound. Association between MIC and MBC values of chlorhexidine and isopropyl alcohol for strains of *K pneumoniae*positive and negative for ESBL production was studied. A significantpositiveassociation($p<0.001$)between theMICandMBCvalueswasfound.

Time Kill Assay: The initial bacterial inoculum used for time-kill assay was 1.5 x 10^8 CFU/ml. The time kill assays for *K pneumoniae* are shown in Table.3. It is clear that undiluted chlorhexidine (2.5%) and undiluted isopropyl alcohol (100%) had better inhibitory effect compared with chlorhexidine at 1 in 2 dilutions (1.25%) and isopropyl alcohol at 1 in 2 dilutions (50%). The viable count of *K pneumoniae* positive and negative for ESBL production decreased as the time advanced. Complete killing of ESBL negative *K pneumoniae* occurred within 4 h of exposure to undiluted chlorhexidine (2.5%) and undiluted isopropyl alcohol (100%). At 1 in 2 dilutions both chlorhexidine and isopropyl alcohol did not show complete killing of ESBL positive and negative *K pneumoniae.*

Statistical analysis of Time kill assay: Correlation between the growth of *K pneumonia* strains at 0, 2 and 4 hrs. of incubation with chlorhexidine (2.5%) and Isopropyl alcohol (100%) at undiluted and 1 in 2 dilution was studied. To find the association in growth at 0 and 2 h of incubation, Pearson's chi-square test was used. A significant positive association ($p<0.001$) was found. Since viability of *K pneumonia* strains was less after 4 hrs. of incubation, Fisher test was used for comparison. A positive association ($p<0.001$) between the time of incubation and growth was found.The activity of the two biocides was compared and chlorhexidine was found to be more effective biocide when compared to isopropyl alcohol. There was a positive association ($p<0.001$) in the time of exposure and the biocide activity.

Biocide	Organism	Number of isolates with MICs (mg/ml)							
		10	5	2.5	1.25	0.625	0.313	0.156	0.078
Chlorhexidine	ESBL producing K pneumoniae (97)	0	0	0	0	21	36	29	11
	Non ESBL producing K pneumoniae (83)	0	0	0	0	0	16	37	30

Table 1: MIC of chlorhexidine for *K pneumoniae* by broth dilution technique

Biocide	Organism	Number of isolates with MICs (mg/ml)							
		15.6	7.8	3.9	1.95	0.975	0.486	0.244	0.122
Isopropyl alcohol	ESBL producing K pneumoniae (97)	10	42	34	9	2	0	0	0
	Non ESBL producing K pneumoniae (83)	0	16	38	24	5	0	0	0

Table 2: MIC of isopropyl alcohol for *K pneumoniae* by broth dilution techniques

Biocides	Organism	Concentration	Percentage viability of *K pneumoniae*		
			0hr	2hr	4hr
Chlorhexidine	ESBL producing *K pneumoniae*	Undiluted (2.5%)	17.5	5.2	1
		1 in 2 dilutions (1.25%)	60.8	25.8	4.1
	Non-ESBL producing *K pneumoniae*	Undiluted (2.5%)	14.5	2.4	0
		1 in 2 dilutions (1.25%)	38.6	15.7	1.2
Isopropyl alcohol	ESBL producing *K pneumoniae*	Undiluted (100%)	38.1	20.6	3.1
		1 in 2 dilutions (50%)	72	51.6	17.2
	Non-ESBL producing *K pneumoniae*	Undiluted (100%)	20.5	1.2	0
		1 in 2 dilutions (50%)	53	24.1	9.6

Table 3: Time Kill Assay results of Chlorhexidine and Isopropyl alcohol on *K pneumoniae*

DISCUSSION:

Multi-drug resistant *K pneumoniae* identified as an urgent threat to human health by the World Health Organization, the US Centres for Disease Control and Prevention and the UK Department of Health. Agar dilution, disk diffusion, micro-broth and macro-broth dilutionare the methods used for testing the sensitivity of microorganism to antibiotics. In the present study the biocide activity of chlorhexidine (2.5%) and isopropyl alcohol (100%) was tested against 180 *K pneumonia* strains. The majority of our isolates were acquired from urine (24%), followed by swabs (22%), then blood (17%) and ET tip (14%) signifying the true infection. The isolated strains of *K pneumoniae*positive for ESBLproduction were resistant to carbapenems, fluoroquinolones, and aminoglycosides. *K pneumoniae* ATCC 700603 (ESBL producer) and *K pneumoniae* ATCC 13883 (non ESBL producer) were used as control strains. The MIC of Chlorhexidine for 97 *K pneumoniae positive* for ESBL production was in the range of 0.078 to 0.625mg/ml and 83 *K pneumoniae*negative for ESBL production was in the range of 0.078 to 0.313mg/ml. The MIC of isopropyl alcohol for *K pneumoniae*positive for ESBL production was in the range of 0.975 to 15.6mg/ml and *K pneumoniae* negative for ESBL production was in the range of 0.975 to 7.8mg/ml. In this study we found that MIC of chlorhexidine and isopropyl alcohol for ESBL positive *K pneumoniae* is higher when compared ESBL negative *K pneumonia* and chlorhexidine (2.5%) was found to be more effective as a biocide when compared to isopropyl alcohol (100%).Earlier studies have reported an increase in carbapenem resistance in *K pneumonia*. Carbapenem resistant *K pneumoniae*has shown to be more resistant to the commonly used biocides when compared to carbapenem sensitive *K pneumoniae*[2]. Studies suggest that using any one biocide may not be effective to eliminate all carbapenem resistant *K pneumoniae*strains [1]. An increase in the time of exposure and the combined uses of more than one biocide may help in complete eradication of the microorganism. This can be also applied in hand hygiene practices, mouth washes etc. Studies show that over exposure to biocides may lead to emergence of reduced susceptibility among *K pneumonia* strains [2]. An increase in time of exposure may enhance the bactericidal activity of biocides. In the current study antimicrobial effect of biocides was determined by time kill assay, Chlorhexidine (2.5%) was found to inhibit growth at lower time of exposure when compared to isopropyl alcohol (100%).

Molecular techniques will be helpful to study the resistance in terms of genetic mechanisms. Amitabha et al; have reported that resistance to tigecycline and polymixin-B in strains of *K pneumoniae* producing carbapenemase. *K pneumoniae* producing New Delhi Metallo β-lactamase has emerged worldwide and infections caused by such strains are difficult to treat because of their pan resistance. The current study has revealed that multi drug resistant *K pneumonia* strains have reduced susceptibility to biocides such as chlorhexidine (2.5 %) and isopropyl alcohol (100%).The study also shows that chlorhexidine is more bactericidal when compared to isopropyl alcohol. The exposure time and bactericidal activity was found to be directly proportional to each other. The resistance towards biocides is less described unlike antibiotic resistance. In this study we found a positive association between drug resistance and decreased susceptibility to biocides.

CONCLUSION:

Monitoring the susceptibility of *K pneumoniae*an important nosocomial pathogen to commonly used biocides in hospitals may help in implementing appropriate and effective infection control measures. The present study revealed the susceptibility pattern of *K pneumoniae* to biocides. By this we can decide the best disinfectants for elimination of ESBL as well as Non ESBL strains of *K pneumonia*. The study suggests that there is positive correlation between the drug resistance and reduced susceptibility towards biocides.

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