Original Research

The susceptibility of multidrug resistant and biofilm forming *Klebsiella pneumoniae* and *Escherichia coli* to antiseptic agents used for preoperative skin preparations at zonal referral hospital in Mwanza, Tanzania

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Abstract

Background

Non-susceptibility of bacteria to antiseptic agents used for preoperative skin preparations threaten the effectiveness of prevention of surgical site infections. Data concerning susceptibility of multidrug resistant bacteria strains to antiseptic agents was limited at our setting. This study presents the susceptibility of extended spectrum β-lactamases producing *Klebsiella pneumoniae* and *Escherichia coli* (with and without biofilm formation) to antiseptic agents used for preoperative skin preparations at zonal referral hospital in Mwanza, Tanzania.

Methods

This cross-sectional descriptive study was conducted through July 2020. Presumptive extended spectrum beta-lactamase producing *Klebsiella pneumoniae* and *Escherichia coli* were recovered for this study. Disc combination method was used to confirm production of ESBL while tube method was used to detect biofilms formation. Then, isolates were tested for susceptibility towards 10% povidone iodine, 70% methylated spirit, 50% hydrogen peroxide (6% of industrial H_2O_2 diluted in equal volume with sterile distilled water) and 2% chlorhexidine. STATA software version 13.0 was used for data analysis.

Results

A total of 31 presumptive ESBL producers were recovered and phenotypically confirmed, whereas 54.8% (n=17) were *K. pneumoniae* and 45.2% (n=14) were *E. coli*. Five (35.7%) *E. coli* and seven (41.2%) *K. pneumoniae* had positive biofilms test results. Four (12.9%) bacteria were non-susceptible to antiseptic agents used for preoperative skin preparations. However, none exhibited resistance towards 10% PVP-I.

Conclusion

In this study we highlight the existence of multidrug resistant Gram-negative bacteria with resistance to antiseptic agents used for preoperative skin preparation at a zonal referral hospital in Mwanza, Tanzania.

Keywords: antiseptic agents; biofilms; extended spectrum beta-lactamases; *Escherichia coli; Klebsiella pneumoniae*; multidrug resistance; surgical site infections.

Introduction

Antiseptics are substance that kills or inhibits the growth of microorganism in or on the living tissue[1,2]. Antiseptics are used for different purposes including preoperative skin preparations and wounds irrigation postoperative procedures, depending on their strength, for the purpose of prevention or management of wound infections particularly surgical site infections (SSIs)[2]. Therefore, efficacious antiseptic agents with broad spectrum and bactericidal activities are recommended for preoperative skin preparations. Aqueous-based solutions (e.g., povidone-iodine (PVP-I) and chlorhexidine gluconate (CHG)) and alcohol-base solutions (e.g., ethyl and isopropyl alcohol) are commonly used in operating theatre rooms[3].

The emergence of non-susceptible bacterial strains to antibiotics and antiseptic agents is reported worldwide[4].

Bacteria develops resistance when exposed to pressure from antibiotics and antiseptic agents contaminating environments[5]. It is also reported that, most bacterial strains exhibiting resistance to multiple antibiotics are also exhibiting resistance to certain antiseptic agents[5]. However, resistance to antiseptic agents can be organism's natural property (intrinsic) which is mediated by impermeability, efflux, biofilms, and enzymatic degradation[2,6]. Organisms may also acquire resistance to antiseptics through chromosomal mutation or acquisition of mobile genetic elements that harbour genes responsible for antiseptics resistance[2,6]. *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus* species, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common bacteria exhibiting resistance to antiseptic agents[2,6].

Gadea *et al*, reported that 88.2% and 30.3% of bacteria strains developed resistance to benzalkonium chloride and

hexadecylpyridinium chloride respectively after exposure to quaternary ammonium compounds[7]. Study by Guimarães et al, reported that 52% and 38% of antibiotic-multiresistant bacteria strains were non-susceptible to quaternary ammonium and phenol compounds respectively[8]. Non-susceptibility of bacteria to antiseptic agents threaten their effectiveness in prevention of SSIs pre- and post-operations. In Mwanza, Tanzania, the incidence of SSIs is ranging from 10.9% to 29.8% among patients with surgical acute abdomen, caesarean section and major surgeries[9]. Non-susceptibility of bacteria to antiseptic agents, among other factors, may be associated with SSIs at this setting. However, data concerning susceptibility of multidrug resistant bacteria strains to antiseptic agents was limited. The first objective of this study was to determine the magnitude of biofilm formation among extended spectrum β-lactamases producing E. coli (ESBL-EC) and K. pneumoniae (ESBL-KP). The second objective was to determine the susceptibility of ESBL-EC and ESBL-KP (with and without biofilm formation) to antiseptic agents used for preoperative skin preparations at zonal referral hospital in Mwanza, Tanzania. Findings from this study provides baseline information to improve infections prevention and control (IPC) guidelines in operating theatres and surgical wards.

Materials and Methods

Study design, period, and setting

This cross-sectional descriptive study was conducted through July 2020 in a Microbiology laboratory, a research, consultancy and teaching laboratory at Catholic University of Health and Allied Sciences-Bugando (CUHAS-Bugando) affiliated in Bugando Medical Centre (BMC), Mwanza-Tanzania.

Antiseptic agents used for this study

Ten millilitre of each antiseptic agent available in operating theatre rooms at BMC ready for use as preoperative skin preparation: 10% povidone iodine (10% PVP-I), 70% methylated spirit (70% MS), 50% hydrogen peroxide (50% H_2O_2; 6% of industrial H_2O_2 diluted in equal volume with sterile distilled water) and 2% chlorhexidine (2% CHX) was collected in sterile, wide mouth and screw capped specimen containers. Specimens were brought to Microbiology laboratory at CUHAS-Bugando for analysis within 30 minutes.

Isolates selection and recovery

Thirty five presumptive extended spectrum beta-lactamase (ESBL) producing Klebsiella pneumoniae (ESBL-KP) and Escherichia coli (ESBL-EC) stored in 20% glycerol in brain heart infusion broth (BHI; CM1135, Oxoid, UK) and archived at -80°C were selected and recovered for this study. Isolates were recovered by sub-culturing on plain plates of MacConkey agar (MCA; CM0109, Oxoid, UK) and then plates were incubated in ambient air at 37°C for 24 hours. Selected bacteria were previously isolated from rectal colonization in a study conducted in neonatal intensive care unit at Bugando Medical Centre in Mwanza, Tanzania. Originally, test bacteria were isolated on MacConkey agar plates which were supplemented with cefotaxime 2μg/ml for the purpose of screening presumptive ESBL producing Gram-negative bacteria (resistant to third generation cephalosporins, 3GCs).

Phenotypic confirmation of ESBL production

A disc combination method for confirmation of ESBL production in E. coli and K. pneumoniae as reported in Clinical and Laboratory Standards Institute (CLSI) guidelines[10] was used in this study. Bacteria were suspended in sterile normal saline 0.85% with turbidity equivalent to 0.5 McFarland standard solution and then plates of Muller Hinton agar (MHA; CM0337, Oxoid, UK) were swabbed to make even lawns. Thereafter, within 15 minutes, ceftazidime 30μg discs (with and without clavulanic acid 10μg) were seeded and plates were incubated in ambient air at 37°C for 18-24 hours. Isolates with increased zone of inhibition of ≥5 mm of ceftazidime with clavulanic acid compared with ceftazidime without clavulanic acid was confirmed as ESBL producer.

Detection of biofilm formation

Tube method as previous reported by Karigoudar et al.,[11] was used for detection of biofilm formation. A loopful (10μl) of test bacteria from overnight cultures were inoculated in 4 ml of tryptic soy broth (TSB; Liofilchem, Italy) containing test tubes and then tubes were incubated aerobically at 37°C for 24 hours. After 24 hours of incubation, tubes were decanted, washed with phosphate buffer saline (PBS, pH 7.3) and allowed to dry in the inverted position at room temperature. The interior of dried tubes were stained with crystal violet 0.1% for 1 minute followed by washing with distilled water to remove excess stain and then dried in the inverted position at room temperature. Each isolate was tested in duplicate to confirm test results. Presence of visible film lining the wall and bottom of the tubes were interpreted as positive biofilm formation and absence of visible film lining the wall and bottom of the tubes were interpreted as negative biofilm formation (Figure 1).
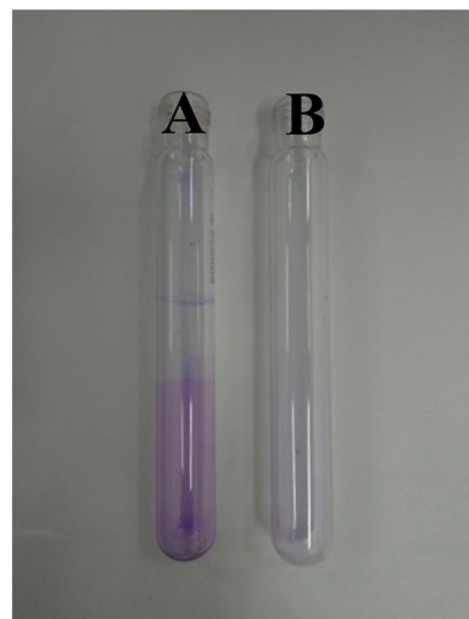

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Testing of susceptibility of bacteria to antiseptic agents

Each strain of bacteria was suspended in two test tubes containing sterile normal saline 0.85% ending with a turbidity equivalent to 0.5 McFarland measured by densimeter (DensiCHEK plus; BioMérieux, USA). Selection of the strength of suspension turbidity was based on the fact that, before application of antiseptic agent(s) before operation,

Table 1: Bacteria strains exhibiting non-susceptibility activity towards antiseptic agents

S/No	Isolate	Biofilm formation	2% CHX		10% PVP-I		50% H$_2$O$_2$		70% MS	
			AD	10 min	AD	10 min	AD	10 min	AD	10 min
1	*E. coli*	Positive	+	-	-	-	-	-	-	-
2	*K. pneumoniae*	Negative	-	-	-	-	-	-	+	-
3	*K. pneumoniae*	Negative	-	-	-	-	+	-	-	-
4	*E. coli*	Positive	+	-	-	-	-	-	+	-
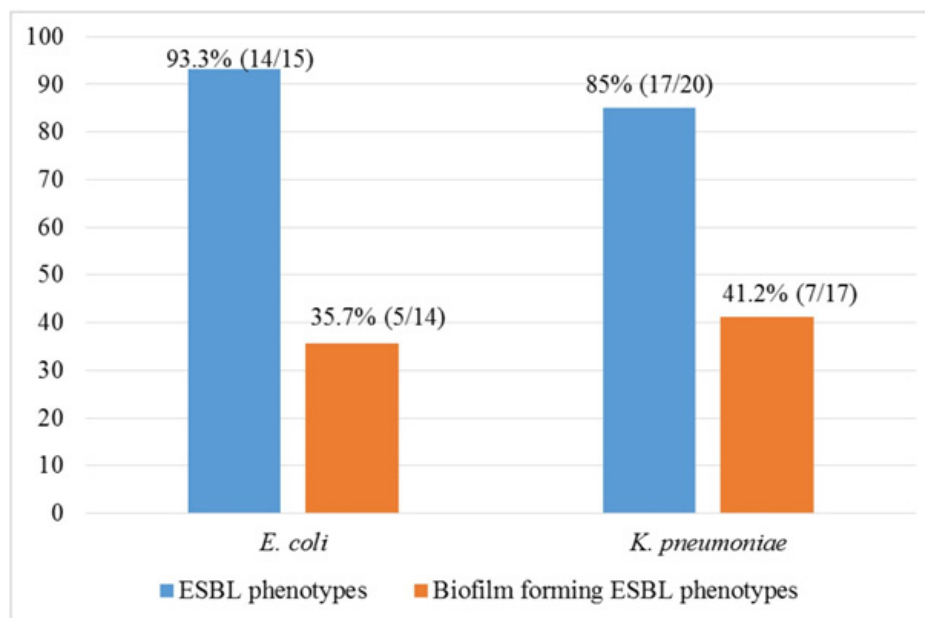
Figure 2: Percentages of phenotypically confirmed ESBL producing *E. coli and K. pneumoniae* and biofilm formation among ESBL-EC and ESBL-KP.

the surface is cleaned with water and detergent. Cleaning minimizes the load of both, resident and transient flora. Sterile cotton swabs (Improswab; Guangzhou, China) were dipped into suspension in each test tube and then swabbed on sterile (autoclaved at 121°C and 15lbs for 15 minutes) surface (a diameter of 3 cm) of flat-bottom flask (Pyrex; Corning Inc, USA) and left for 10 minutes to contaminate the surface. Then, sterile absorbent gauze moistened with antiseptic agent for preoperative skin preparation was used to decontaminate the contaminated surface of flat-bottom flask. To ensure effective decontamination, the area of coverage was extended to a diameter of 6 cm. Two sample swabs were collected by swabbing on decontaminated surface of flat-bottom flask. Firstly, soon after antiseptic agent air dry and secondly, after 10 minutes from antiseptic agent air dry. We chose this method (surface contamination and decontamination) to mimic the procedure of antiseptics application on living tissues.

Swab samples were inoculated in 4 ml of TSB as neutralizing agent and incubated for 4 hours at 37°C in ambient air. After 4 hours of incubation, 10μL of each inoculated TSB was quantitatively sub-cultured on plates of 5% sheep blood agar (Oxoid, UK) and then incubated aerobically for 24 hours at 37°C. Bacteria strains on plates with positive cultures were quantified and identified to make sure the same contaminated bacteria strain is isolated. Biochemical identification of isolates was done by using in-house prepared identification tests; TSI, SIM, Simmons citrate and urease agar. Identification of similar isolate previously used

to contaminate surface of flat-bottom flask was interpreted as non-susceptibility to particular antiseptic agent used for decontamination.

Data analysis

STATA software version 13.0 was used for data analysis. Results are presented in percentages and fractions. PRTest was used to examine statistical difference between ESBL-KP and ESBL-EC or biofilms positive and negative in resisting antiseptic agents.

Ethical considerations

Methodologies of this study were ethically cleared and approved by the joint BMC/CUHAS Ethics & Review Committee with certificate no: CREC 1503/2020. Permissions to conduct this study were sought from respective administrations of Bugando Medical Centre (BMC) and Microbiology laboratory, Catholic University of Health and Allied Sciences-Bugando (CUHAS-Bugando).

Results

Isolates recovery and phenotypic confirmation of ESBL productions

A total of 35 presumptive ESBL producing Gram-negative bacteria (20 *K. pneumoniae* and 15 *E. coli*) were recovered during this study period. Out of 35 presumptive ESBL producing Gram-negative bacteria, only 31; *K. pneumoniae* (85%, 17/20) and *E. coli* (93.3%, 14/15) were phenotypically confirmed to be ESBL producers by CLSI disc combination method.

Phenotypically confirmed ESBL producing *E. coli* and *K. pneumoniae* were further analyzed for biofilm formation and susceptibility to antiseptic agents.

Biofilm formation

Five (35.7%) out of 14 ESBL-EC and seven (41.2%) out of 17 ESBL-KP were positive for biofilm formation on tube method. The difference between the magnitude of biofilm formation of ESBL-EC and ESBL-KP was not significant, p=0.847 (Table 1 and Figure 2).

Susceptibility of test bacteria to antiseptic agents

Four (12.9%) out of 31 ESBL producing Gram-negative bacteria were non-susceptible to antiseptic agents used for preoperative skin preparations. In general, the quantity of bacterial growth on recovery culture plates, as the level of resistance, ranged from 50 to 100 colonies. The overall resistance of ESBL bacteria to individual antiseptic agent was 3.2% (1/31), 3.2% (1/31) and 6.4% (2/31) to 2% CHX, 50% H_2O_2 and 70% MS, respectively. All bacteria strains (100%, n=4) showed resistance to antiseptic agents after air dry but not after exposure of 10 minutes. None of the isolate tested exhibited resistance to 10% PVP-I (Table 1). We observed no significance difference between ESBL-KP and ESBL-EC (p=1.00) or positive and negative biofilm formation (p=1.00) in resistance to antiseptic agents.

Discussion

The main purpose of antiseptic agents used before, during and after invasive procedures such as vein-punctures and minor or major surgeries is to minimize the risks of endemic and epidemic healthcare associated infections (HCAIs)[8]. Surgical site infections (SSIs) and bloodstream infections (BSIs) are some examples of HCAIs associated with ineffective use or the use of substandard antiseptic agents[12]. A wide range of antiseptic agents, aqueous- (e.g., povidone iodine and chlorhexidine gluconate) and alcohol-based (e.g., ethyl and isopropyl alcohol), are approved for clinical use in healthcare settings[3]. At their appropriate concentrations, standard antiseptic agents exhibit bactericidal activity against a wide range of microorganisms[13].

The emergence and effective spreading of antimicrobial resistance among Gram-negative bacteria particularly members of the family Enterobacteriaceae threatens the effectiveness of infectious diseases treatment. It is well established that, significant number of bacteria with resistance to multiple antibiotics are also resistant to antiseptic agents[5]. At the current study's setting, information on the magnitude of extended spectrum β-lactamase producing Gram-negative bacteria (with and without biofilm production) resistant to antiseptic agents was limited. This study aimed at filling the existed gap.

In this study, nearly one half of ESBL-KP were biofilm producers. This observation is similar to report by Nirwati et al.,[14] who reported a prevalence of 49.1% of biofilm production among clinically isolated MDR-*K. pneumoniae*. Contrarily to study by Olowe et al.,[15] who reported a prevalence of 100% of biofilm formation among MDR-*E. coli*, in our study we observed that, about one third of ESBL-EC were biofilm producers. The difference of magnitude of biofilm formation among strains of *E. coli* from study by Olowe et al., and our study could be due to different level of resistance between *E. coli* strains used in the two studies. As Olowe et al., used MDR *E. coli* strains (resistant to multiple antibiotics of different chemical structures, and site and mechanisms of actions) while in our study we used ESBL *E. coli* strains (resistant to β-lactams). Biofilm forming MDR bacteria are associated with resistance to multiple antibiotics compared to their counter-parts. Therefore, increasing the risk of patients' treatment complications including treatment failure then increased mortality.

In consistence to previous studies[16,17], this current study also reports non-susceptibility (12.9%) of ESBL producing Gram-negative bacteria (with and without biofilm formation) to antiseptic agents commonly used as preoperative skin preparations; 2% CHX, 50% H_2O_2 and 70% MS at our setting. Chen et al.,[16] reported a reduced susceptibility of carbapenemases producing *K. pneumoniae* and *E. coli* to 0.1% CHX by MIC_{90} 32mg/L and 16mg/L respectively. In the study by Chen et al., all of the isolates (100%) tested had reduced susceptibility to antiseptic agents while in our study about one in ten (12.9%) of the isolates tested exhibited resistance to antiseptic agents. This difference could be because, not only Chen et al., used bacteria strains with superior resistance mechanisms (carbapenemases production) to ours (ESBLs producers) but also they used low concentrations of antiseptic agents compared to ours.

Another study by Liu et al., reported a reduced susceptibility of all carbapenems resistant *Acinetobacter* baumannii tested to CHX ranging from 4 to 64 µg l^{-1} [18].

Although Liu et al., used known carbapenems resistant strains of Acinetobacter baumannii a bacterium known with its general innate low susceptibility to multiple antibiotics and antiseptics[19] as compared to bacteria strains used in our study. Therefore explaining the highest magnitude of resistance observed in a study by Liu et al., compared to our study.

Similarly to reports by Cochran et al.,[20] and Perumal et al.,[21] we also observed non-susceptibility of tested bacteria to 50% H_2O_2. But, Cochran et al., and Perumal et al., demonstrated an increased reduced susceptibility among biofilm forming cells than planktonic cells while we observed resistance to 50% H_2O_2 among planktonic cells of ESBL-KP. Although biofilm formation is known to increase resistance to antiseptic agents, the planktonic cells of ESBL-KP in our study may have had acquired another mechanism of resistance towards H_2O_2. In alignment to a study by Awodele et al.,[22] we observed non-susceptibility of ESBL-EC (with positive biofilms) and ESBL-KP (with negative biofilms) to 70% MS. However, Awodele et al., observed resistance at 50% MS but not at 100% MS. A review by Bigliardi et al.,[23] reported weak bactericidal activity of alcohol-based antiseptic particularly 70% ethanol towards Gram-negative bacteria.

From our findings, the presence of ESBL producing Gram-negative bacteria (with and without biofilm production) resistant to antiseptic agents used for preoperative skin preparations, suggest a possible source of SSIs at this setting. A surveillance by Moremi et al., reported a prevalence of 14.6% SSIs at the same region[24]. However, that surveillance found no proof of hospital's water plumbing system and rectal carriage playing the role of transmission[24]. Therefore, pointing to another sources that may include strains of bacteria colonizing or contaminating at incision site which are resistant to antiseptic agents used for preoperative skin preparation and postoperative surgical site dressing.

In this study we observed no bacteria with resistance to 10% PVP-I. This observation align with a review by Bigliardi et

al., that PVP-I lack resistance and it is efficacious against biofilms[23]. PVP-I is considered the most effective antiseptic agent as it has the broadest spectrum of antimicrobial activity compared to other available antiseptic agents[25,26]. Therefore, 10% PVP-I may be considered as first-choice antiseptic agent at our setting as long as prevention and treatment of SSIs is concerned, as reported previous[25].

Moreover, we observed that, all ESBL-KP and ESBL-EC with and without biofilms with resistance to antiseptic agents, exhibited resistance only after antiseptic agent air dry but not after a 10 minutes exposure. Increased exposure time, significantly increases antimicrobial activity of a particular antiseptic agent[25]. For example, with increased exposure time, PVP-I is said to exhibit activity against spores and some viruses including influenza virus[25,27]. Therefore, increasing time of exposure for an antiseptic agent for a better outcome is mandatory.

Conclusion

In this study we highlight the existence of multidrug resistant Gram-negative bacteria (with and without biofilms) exhibiting resistance to antiseptic agents used for preoperative skin preparation at a zonal referral hospital in Mwanza, Tanzania. We recommend further studies to explore the direct association between antiseptics resistant bacteria and the incidence of SSIs at our setting. We also recommend molecular studies to determine antiseptic resistance genes circulating among clinical and environmental isolates in our setting.

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Authors' contributions

PD, EJS and VS designed the study; EJS collected data; PD, EJS and VS performed laboratory procedures; VS wrote the first draft of this manuscript which was critically revised and approved by all authors.

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Conflicts of interest

Authors declare no conflicts of interest.

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