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Benzalkonium chloride effectiveness as a disinfectant against hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA)

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ABSTRACT

Aims: Benzalkonium chloride is used to disinfect hospital instruments to prevent nosocomial infection caused by microorganisms, such as Methicillin Resistant *Staphylococcus aureus* (MRSA). There are strains of MRSA isolated from hospitals that were found to be resistant towards benzalkonium chloride. This research was aimed to compare the affectivity of different concentrations of benzalkonium chloride to inhibit the growth of Hospital-Associated MRSA (HA-MRSA) and determine the Minimum Inhibitory Concentration (MIC) of benzalkonium chloride against HA-MRSA.
Methodology and results: The samples were five HA-MRSA isolates obtained from Dr. Soetomo Hospital Surabaya. It was identified by amplification of SCC*mec* genes. The HA-MRSA with SCC*mec* type III was divided into six flasks based on the concentration of benzalkonium chloride in their inoculation media (0 µg/mL, 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, and 10 µg/mL). The growth of HA-MRSA in media was determined by the number of colonies after treatment. The result showed that the MIC of benzalkonium chloride for HA-MRSA was 5 µg/mL, where no growth of bacterial was observed. There was significant difference in MRSA colony count between different groups of benzalkonium chloride concentrations (p = 0.001), and there was negative correlation between benzalkonium chloride concentration and HA-MRSA growth (p = 0.0001 and r = -0.880).
Conclusion, significance and impact of study: The concentration of benzalkonium chloride influences the growth of HA-MRSA. The higher the concentration, the fewer HA-MRSA growth. Application of benzalkonium chloride according to MIC will prevent HA-MRSA resistance towards benzalkonium chloride.

Keywords: HA-MRSA, benzalkonium chloride, concentration, MIC

INTRODUCTION

Benzalkonium chloride is a disinfectant used to prevent the spread of nosocomial infections through hospital instruments (McDonnel and Russell, 1999; Fazlara and Ekhtelat, 2012). Benzalkonium chloride is a Quaternary Ammonium Compound (QAC) that works as a disinfectant by damaging the phospholipid bilayer of the bacterial cell, then entering into the cell and denaturing the essential proteins and inactivating the metabolic enzymes required by the cell. The destruction of cell and enzyme proteins then causes cell death (Rutala and Weber, 2008).

Hospital-Associated Methicillin Resistant *Staphylococcus aureus* (HA-MRSA) is one of the bacteria that cause nosocomial infections (Gordon and Lowy, 2008). The source of MRSA transmission can be from carrier individuals or contaminated instruments (Otto, 2012; Watkins *et al*., 2012; Bellows *et al.*, 2013). MRSA is a *S. aureus* strain that is resistant to methicillin and other β-lactam class antibiotics (e.g. penicillin and cephalosporin) (Todar *et al.*, 2000). MRSA is resistant to methicillin because it has a *mecA* gene which is located in a large chromosome element called SCC*mec*. The mode of resistance of MRSA to antibiotics is a change in Penicillin Binding Protein (PBP) so that it is resistant to beta-lactam class antibiotics (Watkins *et al*., 2012). The most common HA-MRSA causing nosocomial infection is Staphylococcal Cassette Chromosome *mecA* (SCC*mec*) type III, which composed 94.4% of all the HA-MRSA (Ashgar, 2014; Santosaningsih *et al.*, 2014), and 98% in Dr. Soetomo Hospital Surabaya (Lelitawati, 2016). Because of its high prevalence, the HA-MRSA type which is used in this research is HA-MRSA with SCC*mec* type III.

Benzalkonium chloride is generally used as a disinfectant for hospital instruments to rid them of

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microorganisms, such as MRSA (Al-Masaudi *et al.*, 1988). However, there have been reported MRSA strains resistant to benzalkonium chloride which were isolated from Kyushu University Hospital, Japan (Akimitsu *et al*., 1999). Meanwhile, there is lack of study concerning the presence of MRSA strains, especially HA-MRSA (including type III), which are resistant to benzalkonium chloride in Indonesia. This study determined the effectiveness of benzalkonium chloride in various concentrations as disinfectant against HA-MRSA with SCC*mec* type III.

MATERIALS AND METHODS

Ethical clearance

Ethical clearance for this research was obtained from Ethical Committee of Faculty of Medicine Universitas Airlangga with the ethical clearance certificate number 183/EC/KEPK/FKUA/2016.

Bacterial isolates

Five samples of MRSA bacterial isolates were obtained from the isolates taken from the patients who were admitted to Dr. Soetomo Hospital Surabaya with MRSA infection and then stored in the Laboratory of Clinical Microbiology (No. 183/EC/KEPK/FKUA/2016).

Determination of SCC*mec* type

To identify HA-MRSA with SCC*mec* type III, a confirmatory test was performed using multiplex PCR method (Milheirico *et al.*, 2007). Five samples of MRSA with isolate number 448, 575, 646, 686 and 700 were cultured on slant agar medium, then sent to Microbiology Laboratory of Medical Faculty of Brawijaya University for DNA isolation and Polymerase Chain Reaction (PCR).

There were 3 primer pairs used in this experiment, (1) *mecA* primer pair for detecting *mecA* gene found in MRSA with 162 base pairs of amplicon, with *mecA* P4: TCCAGATTACAACTTCACCAGG and *mecA* P7: CCACTTCATATCTTGTAACG; (2) *mecI* primer pair for detecting *mec* complex found in HA-MRSA SCC*mec* type II and III with 209 base pairs of amplicon, with *mecI* P2: ATCAAGACTTGCATTCAGGC and *mecI* P3: GCGGTTTCAATTCACTTGTC; and (3) SCC*mec* III primer pair for detecting J1 region found in HA-MRSA SCC*mec* type III with 243 base pairs of amplicon, with SCC*mec* III J1 F: CATTTGTGAAACACAGTACG and SCC*mec* III J1 R: GTTATTGAGACTCCTAAAGC.

The HA-MRSA SCC*mec* type III identification method above was based on methods used in Murakami *et al.* (1991), Zhang *et al.* (2005) and Milheirico *et al.* (2007). The reagent used for DNA isolation was obtained from iNtRON Biotechnology, Korea. PCR was performed using GoTaq Green Master Mix reagent (Promega, United States of America) and processed with a thermocycler from Bio-Rad, United States of America.

Preparation of medium containing benzalkonium chloride

A 0.1 % solution of benzalkonium chloride was prepared by mixing 10 µL benzalkonium chloride (80%) from Brataco, Indonesia and 7990 µL aquadest into the test tube, then the solution was mixed using micropipette. The amount of 0.1% benzalkonium chloride used for each concentration is 0 µL for 0 µg/mL as a positive control (group 1), 12.5 µL for 0.625 µg/mL (group 2), 25 µL for 1.25 µg/mL (group 3), 50 µL for two media, which are 2.5 µg/mL (group 4) and as a negative control (group 7), 100 µL for 5 µg/mL (group 5), 200 µL for 10 µg/mL (group 6). Six groups were inoculated with MRSA (groups 1-6) and one group is not (group 7). All media were prepared by mixing 0.1% benzalkonium chloride according to the required amount with previously sterilized liquid nutrient agar in the Petri dish with the final volume of medium 20 mL. The Petri dish was then placed in room temperature (25 °C) for 15 min until the nutrient agar was solidified and then incubated in 37 °C for 24 h (Akimitsu *et al*., 1999).

Preparation of bacterial suspension

The colony of HA-MRSA isolate number 646 was taken from agar plate with a sterile loop needle and suspended into a nutrient broth medium in a test tube until it reached 0.5 McFarland turbidity, then the suspension was mixed using vortex. The homogeneous suspension was further diluted six times by 4 mL dilution in nutrient broth medium (final concentration of bacteria: Approx. 10^6-10^7 CFU/mL) (Garcia, 2010).

Inoculation of bacteria

Fifty microliter suspension of diluted bacteria was dripped using micropipette onto the agar medium that has been solidified in a Petri dish (except the medium for negative control). The bacterial suspension was then spread using a sterile spreader. The bacterial-inoculated medium was kept at room temperature until the bacterial suspension on the medium dried, then incubated at 37 °C for 24 h (Akimitsu *et al*., 1999). Four replications were performed on each concentration of benzalkonium chloride that was tested.

Counting colonies

Colony counting was done using BZG 40 colony counter from Hoskin Scientific, Canada. Due to the non-scaling calculations (in some of the Petri dish, the bacteria formed a fog-like growth, so the number of colonies could not be counted), the results of the colony count were converted to ordinal data, with 0 representing 0 colonies from 50 µL of suspension (0 CFU/mL), +1 representing 10-100 colonies from 50 µL of suspension (200-2,000 CFU/mL), +2 representing 100-1,000 colonies from 50 µL of suspension (2,000-20,000 CFU/mL), and +3 representing more than 1,000 colonies from 50 µL of

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suspension (>20,000 CFU/mL) or fog-like growth (Brugger *et al*., 2012).

MIC determination

The effectiveness of benzalkonium chloride as a disinfectant against HA-MRSA was calculated in the number of colonies grown on each agar nutrient medium. The Minimum Inhibitory Concentration (MIC) was determined by the result of the colony counting. MIC of benzalkonium chloride against MRSA was the concentration of benzalkonium chloride in the medium with zero colonies of MRSA growth (Akimitsu *et al*., 1999).

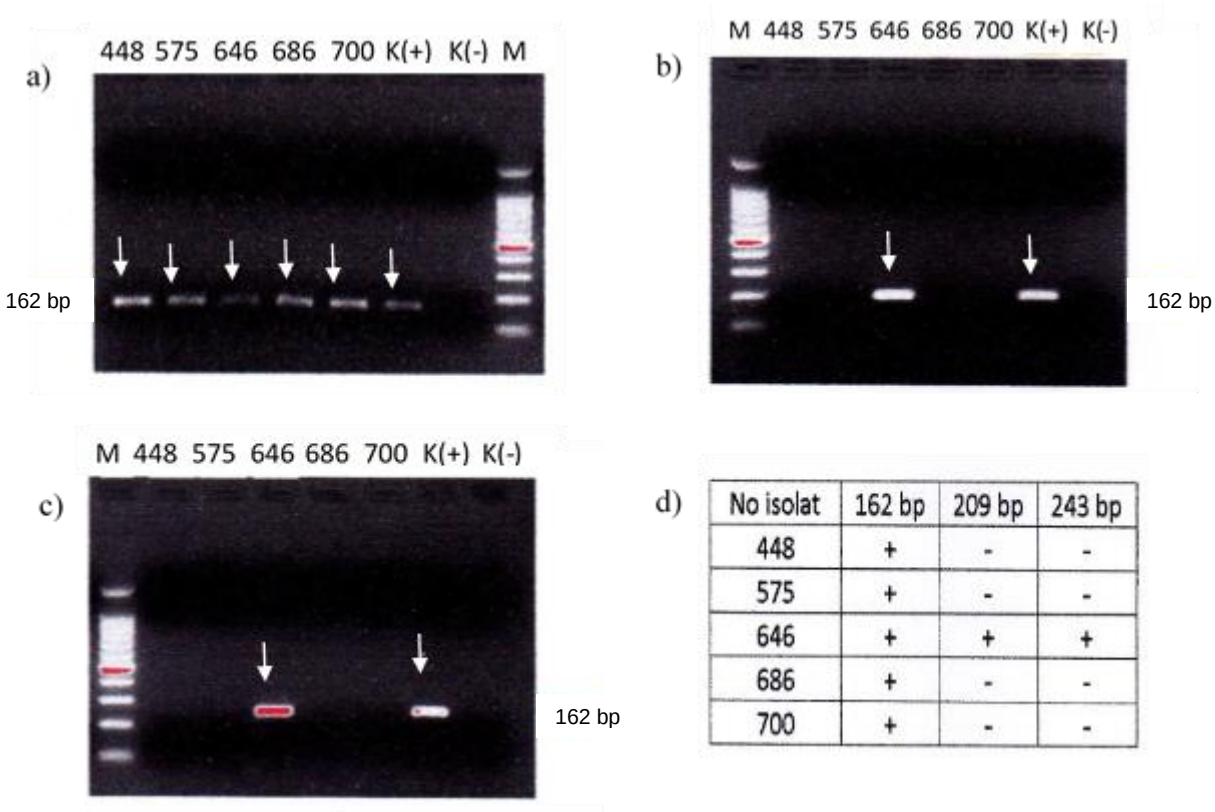
Data analysis

Data which was obtained from seven groups was processed using IBM SPPS Statistics 23 with Kruskall-Wallis comparative test to determine the difference of HA-MRSA growth on media with different benzalkonium chloride concentration. Spearman correlation test was used to find out the correlation between benzalkonium chloride concentration and HA-MRSA growth.

RESULTS

HA-MRSA identification

There were five isolates tested, isolates numbered 448, 575, 646, 686, and 700. Results of PCR of those 5 MRSA isolates are shown in Figure 1. *mecA* gene was positive in isolates 448, 575, 646, 686, and 700, confirming that all of those isolates are MRSA. SCC*mec* type II and III was positive in isolate number 646. PCR HA-MRSA with a target DNA fragment with a length of 243 base pairs (detection of SCC*mec* type III) was positive in isolate number 646.



No isolat	162 bp	209 bp	243 bp
448	+	-	-
575	+	-	-
646	+	+	+
686	+	-	-
700	+	-	-

Figure 1: Results of HA-MRSA SCC*mec* type III PCR visualization with gel electrophoresis. (a) PCR HA-MRSA with a target DNA fragment with a length of 162 base pairs (detection of *mecA* gene), positive in isolates 448, 575, 646, 686, and 700. (b) PCR HA-MRSA with target DNA fragment with length of 209 base pairs (detection of SCC*mec* type II and III), positive in isolate number 646. (c) PCR HA-MRSA with a target DNA fragment with a length of 243 base pairs (detection of SCC*mec* type III), positive in isolate number 646. (d) Resume of the PCR result from each electrophoresis shown in a, b, and c. The isolate that gave positive band in all PCR test, thus identified as HA-MRSA type III, was isolate 646.

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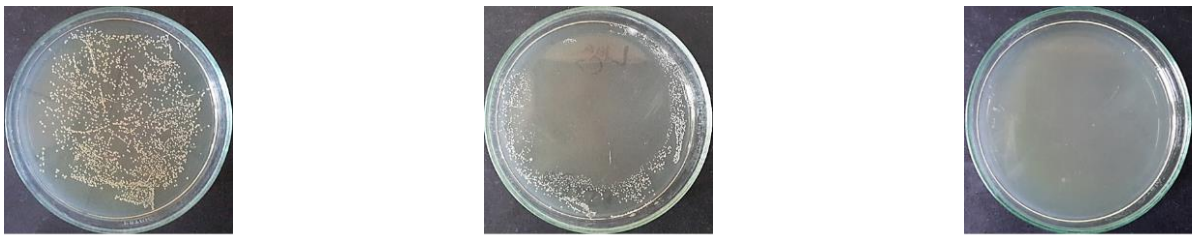

a) b) c)

d)	0 µg/mL or Control (+)	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL	10 µg/mL	Control (-)
Replication 1	+3	+3	+2	+2	0	0	0
Replication 2	+3	+3	+2	+2	0	0	0
Replication 3	+3	+3	0	0	0	0	0
Replication 4	+3	+3	+2	0	0	0	0

Figure 2: Results of HA-MRSA growth on nutrient agar medium containing different concentrations of benzalkonium chloride. (a) Medium showing bacterial growth of +3 [observed at positive control (group 1) and 0.625 µg/mL (group 2) of benzalkonium chloride]. (b) Medium showing bacterial growth of +2 [observed at 1.25 µg/mL (group 3) and 2.5 µg/mL (group 4) of benzalkonium chloride]. (c) Medium showing no bacterial growth [observed at 5 µg/mL (group 5) and 10 µg/mL (group 6) of benzalkonium chloride, also negative control (group 7)]. (d) Resume of the bacterial colony growth after converted to ordinal data that shows the MIC of benzalkonium chloride against HA-MRSA, which is 5 µg/mL.

Table 1: Log of number of bacterial colonies which grew on media with different concentrations of benzalkonium chloride.

Benzalkonium chloride concentration		Log of number of colonies				
		1st replication	2nd replication	3rd replication	4th replication	Mean value
0 µg/mL (Control (+))	log(CFU/50 µL)	3.258397804	3.32056168	3.548757829	3.638788667	3.469674773
	log(CFU/mL)	4.5594278	4.621591676	4.849787824	4.939818663	4.770704768
0.625 µg/mL	log(CFU/50 µL)	3.384532615	Undetermined*	3.46834733	3.56937391	3.480725379
	log(CFU/mL)	4.681964459	Undetermined*	4.769377326	4.870403905	4.780749231
1.25 µg/mL	log(CFU/50 µL)	2.930949031	2.557507202	0	2.909556029	2.705007959
	log(CFU/mL)	4.231979027	3.858537198	0	4.210586025	4.005609445
2.5 µg/mL	log(CFU/50 µL)	2.195899652	2.471291711	0	0	2.053078443
	log(CFU/mL)	3.496929648	3.772321707	0	0	3.355068206
5 µg/mL	log(CFU/50 µL)	0	0	0	0	0
	log(CFU/mL)	0	0	0	0	0
10 µg/mL	log(CFU/50 µL)	0	0	0	0	0
	log(CFU/mL)	0	0	0	0	0
Control (-)	log(CFU/50 µL)	0	0	0	0	0
	log(CFU/mL)	0	0	0	0	0

Benzalkonium chloride sensitivity test

Isolate number 646 was then tested for its sensitivity to benzalkonium chloride. The results of HA-MRSA growth on nutrient agar medium containing six different concentrations of benzalkonium chloride is shown in Figure 2.

The number of HA-MRSA colonies which grew on each nutrient agar medium is shown in Table 1. The number of colonies was then converted to ordinal data and is shown in Figure 2. The graph showing the mean values of the number of HA-MRSA colonies which grew on each nutrient agar medium is shown in Figure 3.

Figure 2, Table 1, and Figure 3 show that the MIC of benzalkonium chloride for HA-MRSA with SCC*mec* type III was 5 µg/mL because benzalkonium chloride in that concentration was effective to to kill all bacteria inoculated in the media.

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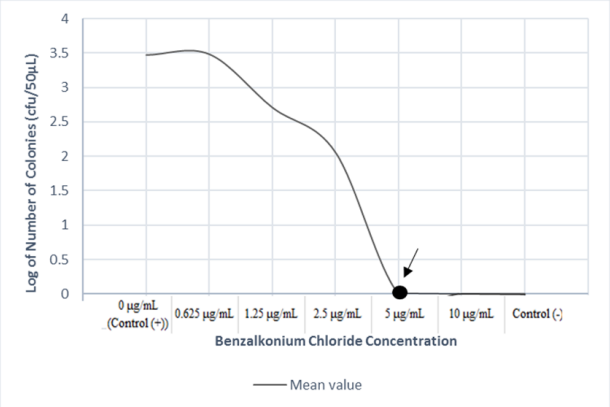

Figure 3: Mean value of the log of number of bacterial colonies (in CFU/50 µL) which grew on each medium containing different benzalkonium chloride concentration. Dot at 5 µg/mL indicated the MIC of benzalkonium chloride against HA-MRSA which was tested.

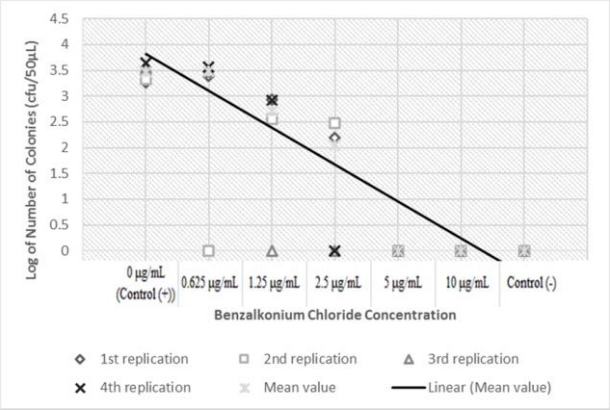

Figure 4: Log of number of bacterial colonies (in CFU/50 µL) which grew on each medium containing different benzalkonium chloride concentration. Linear trendline (black) hows negative correlation between concentration of benzalkonium chloride and the growth of HA-MRSA.

There is significant difference of the number of HA-MRSA growth on the media with different concentrations of benzalkonium chloride, evidenced by the significance value of 0.001 (p <0.05) by Kruskall-Wallis test.

There is a significant relationship between the concentration of benzalkonium chloride with the growth of HA-MRSA when analyzed by Spearman correlation test. It showed the value of significance at 0.0001 (p<0.05). The correlation coefficient obtained was -0.880, which means the negative correlation between the two variables was strong (>0.75 or <-0.75). The higher concentration of benzalkonium chloride will decrease the growth of HA-MRSA. The negative correlation is shown in Figure 4.

DISCUSSION

Minimum Inhibitory Concentration (MIC) of benzalkonium chloride against HA-MRSA isolate with SCC*mec* type III obtained from Dr. Soetomo Hospital Surabaya was 5 µg/mL. This MIC was similar to the MIC of benzalkonium chloride against benzalkonium chloride-sensitive MRSA in a research conducted in Japan by Akimitsu in 1999. According to the research, HA-MRSA with SCC*mec* type III used in this study had no resistance to benzalkonium chloride but has definitely been resistant to antibiotic methicillin because it has *mec*A gene that encodes resistance to methicillin in all MRSAs.

Previous tests of HA-MRSA isolated from various hospitals in Rome, Italy, resulted in benzalkonium chloride having different MICs on the various HA-MRSA strains tested. The MICs were 2 µg/mL, 4 µg/mL, 8 µg/mL, and 16 µg/mL (Raggi and Filippini, 2013). These results suggested that different gene mutations between different HA-MRSA strains may also affect the resistance of HA-MRSA to benzalkonium chloride. This is the reason why this research is important to be conducted.

Research on the effectiveness of benzalkonium chloride as a disinfectant against MRSA has been done before. From these studies, it can be observed that MRSA resistance to benzalkonium chloride is associated with MRSA resistance to certain antibiotics (Fatholahzadeh *et al.*, 2008; Ahmad *et al.*, 2013). This happens because both have the same resistance mechanism, that is through the efflux pump encoded by the same gene (Ubarretxena-Belandia, 2003; Weinstein *et al*., 2005; Quistgaard *et al.*, 2016). Mutation of genes that cause MRSA resistance to benzalkonium chloride is a mutation of the *fem*A gene that also causes resistance to methicillin and oxacillin (Akimitsu *et al*., 1999), as well as *qac* gene mutations that also lead to erythromycin, penicillin and ampicillin resistance (He *et al*., 2014). From the studies it can be concluded that MRSA resistance mechanisms to various types of antibiotics and disinfectants, especially benzalkonium chloride, are not only caused by mutations of one particular gene, but there are several gene mutations that play a role (Reiter *et al*., 2010). Gene mutations in MRSA that cause resistance towards antibiotics are influenced by environmental condition and frequent use of disinfectants (He *et al*., 2014). The MIC of benzalkonium chloride against HA-MRSA might be different at places other than health care institutions, because the MIC is also influenced by previous benzalkonium chloride exposure towards the microorganism (He *et al*., 2014). Thus, the MIC of benzalkonium chloride against MRSA at places other than health care institutions are probably different than those at health care institutions which use benzalkonium chloride frequently as disinfectant.

Different types of MRSA and MRSA growing in different environments are likely to have different gene mutations and patterns of resistance to different antibiotics and disinfectants. This is the reason why researches of effectivity of benzalkonium chloride against HA-MRSA has been done in various countries and

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environments using many different types of MRSA, including in Indonesia using HA-MRSA with SCC*mec* type III as was done in this research.

Contamination of hospital equipment by HA-MRSA will cause nosocomial infection if those contaminated equipment are exposed to the patients. Patients with nosocomial infection caused by HA-MRSA will be difficult to treat using antibiotics, because HA-MRSA has been proven resistant towards numerous antibiotics, such as methicillin. Thus, the application of benzalkonium chloride as disinfectant is important to prevent the nosocomial infection caused by methicillin resistant bacteria (Mollema *et al*., 2010; Otto, 2012).

There are some limitations in this study. First, this research only used one strain of MRSA, which is HA-MRSA with SCC*mec* type III, and second, this study was only conducted at Dr. Soetomo Hospital, Surabaya. However, the result of this research can be used as initial data for further studies concerning MRSA resistance towards benzalkonium chloride in Indonesia.

CONCLUSION

In conclusion, HA-MRSA isolated from Dr. Soetomo Hospital Surabaya is still sensitive to benzalkonium chloride with concentration 5 µg/mL. This concentration can be implemented not only at Dr. Soetomo Hospital Surabaya, but also at another health care institutions in and around Surabaya.

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