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Evaluation of a Hand Antiseptic with WHO-Recommended Formulation and Its Efficacy in Killing Methicillin-Resistant Staphylococcus Aureus (MRSA)

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ABSTRACT

The present pandemic that is caused by COVID-19 and previously by the clone of the methicillin-resistant staphylococcus aureus (MRSA) has threatened human life. This condition requires materials that can break the chain of transmission from human to human and from the environment to human. This study aimed to evaluate the quality of alcohol-based hand antiseptic using WHO-recommended formulation based on the stability of the formulation, the risk of irritation, and the ability to kill bacteria. Assessments on the presence of rancidity, clarity, discoloration, final alcohol content, and skin irritation risk were done to know the quality of the product. Methicillin-resistant staphylococcus aureus (MRSA) was used to assess the percentage of bacterial killing power. The selected bacteria were bacteria that are commonly found in the hospital environment. The results showed that from four variants tested, MK.IV had good stability compared to other formulations. In terms of irritation risk, twenty-three selected subjects could well tolerate the formula. The results of the killing efficacy against MRSA showed that the antiseptic could kill 99.90% of the bacteria at the 1st, 2nd, and 5th minute. A selected manufacturer's product also showed the same killing efficacy at the 1st, 2nd, and 5th minute. The effective value of the antiseptic for each contact time was ≥ 90%.

Keywords: hand rub, hand sanitizer, Ahmad Subhan, bacterial killing efficacy, WHO

INTRODUCTION

At present, infections widespread in society have threatened human life in the world. Coronavirus infection which was introduced by the World Health Organization (WHO) as COVID-19 (Coronavirus disease 2019) has caused massive death until today (WHO, 2020). In Indonesia, Covid-19 infection raised to pandemic events on the 164th day and it has already been 59.394 people who are positively infected with 2.987 people died and 26.667 people recovered (Kemenkes R.I., 2020).

There was once another pandemic caused by *S. aureus* happened in the world. This pathogen was resistant to penicillin in phage type 80/81 which was the most numerous and extraordinary clones causing epidemics during the 1950s. This clone quickly spread and was dominant in Australia, England, America, and Canada, which caused severe skin infections, sepsis, and/or pneumonia. Initially, this pandemic was limited to the hospital environment. However, this infection gradually spread to people outside the hospital. This pandemic lasted for about 10 years after a decline of phage-type 80/81 was observed. The decline was caused by the introduction of methicillin to the market from 2000 to 2006 in Queensland, Eastern Australia. Population studies of antibiotic resistance profile of MRSA in in-patients showed an increase from 71 to 315 cases per million for non-MDR (Multiple Drug Resistance) types. This strain was resistant to at least one non-lactam antibiotic and was susceptible to ciprofloxacin. During the same period, parallel increases were seen in out-patient units, from 52 to 490 cases per million. This study proposed the rapid spread of non-MDR MRSA

strains. Very high MRSA prevalence rates had also been detected in East Asia. Multinational study centers conducted surveillance studies in 2011 and determined the prevalence of MRSA in various Asian countries. They concluded that HA-MRSA was responsible for 86.5% in Sri Lanka, 74.1% in Vietnam, 77.6% in South Korea, 65% in Taiwan, 57% in Thailand, and 56.8% in Hong Kong. However, the prevalence is much lower in India and the Philippines, respectively, 22.6% and 38.1% (Lakhundi and Zhang, 2018).

Pathogen transmission can occur during the process of patient care in the hospital, either through direct or indirect contact as well as droplets or contaminated air. Transmission through contaminated hands is the most common pattern in most health services. For this reason, WHO (2009) established five conditions that required health workers to wash their hands (1) after making contact with patients because the organisms on the patient's skin can move into health workers' hand whenever there is a direct contact; (2) after being exposed to body fluids because pathogenic organisms in body fluids can contaminate officers' hand and thus can be transmitted to patients or vice versa; (3) after direct contact with the patient's immediate environment because the patient's immediate environment has been contaminated by patient's droplet or body fluids; (4) before contact with patients to prevent the transmission of pathogens from the hands of the officer to the patient; (5) before carrying out sterile actions because pathogenic organisms are able to survive for at least a few minutes on officers' hands. Therefore, before carrying out sterile actions, hand rubbing using antiseptic must be done (WHO., 2009).

Antiseptics and disinfectants are widely used in hospitals and other health care facilities for various purposes to protect surface areas. In particular, this is an important part of infection control and is a tangible form of prevention of nosocomial infections. The emergence of concerns about the potential for microbial contamination and the risk of infection in food and other consumption materials, has led to an increase in the use of antiseptics and disinfectants by the mass public. A variety of active chemical agents (or biocides) is found in these products, many of which have been used for hundreds of years for antiseptics, disinfection, and preservation. However, little is known about how this active ingredient works, compared to antibiotics. In general, the biocide has a broader spectrum of activity than antibiotics. Besides, antibiotics tend to have specific intracellular targets, while biocide may have many targets. The widespread use of antiseptic products and disinfectants has led to some speculation about the development of microbial resistance, especially cross-resistance to antibiotics (WHO., 2009)

In addition, the high price of hand antiseptics can be a trigger for the scarcity of it in health care facilities. Based on monthly reports on the use of pharmaceutical supplies in Fatmawati General Hospital, it is known that from May 2016 to September 2018, there were 23,129 bottles in the volume of 500 mL that had been used. The total cost needed was IDR 2,823,000,000 (Subhan and Wasmen, *et al*., 2019).

For this reason, research needs to be carried out as an effort to control pathogens by conducting studies on the quality of the WHO-recommended hand antiseptic formula. The results of this study may be useful to fulfill health care needs in Indonesia. It also may be used as an input for all health services in Indonesia in making a hand antiseptic that is affordable and has a good quality in order to create good and affordable health services for communities. It may increase the repertoire of knowledge that can be applied in health care services in Indonesia.

Hand antiseptic

Alcohol-based hand antiseptics often contain ethanol, isopropanol, n-propanol, or a combination of these. In general, isopropanol has greater bacterial killing efficacy and ethanol is more potential against viruses, but it also depends on the concentration of two active substances and the microorganism test. For example, isopropanol is more lipophilic than ethanol and has less activity against hydrophilic viruses (e.g. poly viruses) (WHO., 2009).

Ethanol (also called ethyl alcohol, granular alcohol, drinking alcohol, or just alcohol) is a chemical compound, a simple alcohol with the chemical formula C_2H_5OH. The formula can also be written as CH_3-CH_2-OH (ethyl group associated with the hydroxyl group) and is often abbreviated as EtOH. Ethanol is a volatile, flammable, colorless liquid with a slight odor. It is a psychoactive substance and a main active ingredient found in alcoholic drink (Haynes and William, 2011).

About 60-80% alcohol is the most effective solution in killing microorganisms. The greater concentration of the alcohol yields the smaller potency of the microorganism to keep optimally

infecting. There is still little information about the specific mechanism of alcohol in killing bacteria, but based on the increased efficacy in the presence of water, in general, alcohol causes membrane damage and protein denaturation, thereby further causes metabolic disorders and protein lysis (Larson and Morton, 1991).

Alcohol damages the cytoplasmic membrane thus it causes intracellular constituent leakage (Fanning S., 2011). Alcohol has excellent germicidal activity *in vitro* against gram-positive and gram-negative vegetative bacteria (including multidrug-resistant pathogens such as MRSA and VRE), *M. tuberculosis*, and varieties of fungi. However, alcohol has virtually no activity against bacterial spores or protozoan oocytes and has poor activity against some non-enveloped (non-lipophilic) viruses. Some enveloped (lipophilic) viruses such as the herpes simplex virus (HSV), HIV, Influenza virus, RSV, and vaccinia virus are sensitive to alcohol when tested *in vitro*. Other enveloped viruses are less sensitive to alcohol, but some studies showed that 60-70% of alcohol can be lysed including hepatitis B virus (HBV) and possibly hepatitis C virus. In a carrier model of porcine tissue used to study antiseptic activity, 70% ethanol and 70% isopropanol were found to reduce the titer of enveloped bacteriophages more effectively than antibacterial soap containing 4% CHG (WHO., 2009). Alcohol quickly kills bacteria when applied to the skin and it has no persistent activity. However, bacterial regrowth in the skin occurs slowly after the use of alcohol-based antiseptic. This is possible because of a sub-lethal effect of alcohol on some skin bacteria (WHO.,2009).

A number of studies have documented the antimicrobial activity of alcohol *in vivo*. Early quantitative studies of the antiseptic effect of hand rub determined that alcohol effectively reduced the number of bacteria on the hands. Typically, there is a reduction in the log of bacteria from artificially contaminated hands that is 3.5 $\log_{10}$ on average after 30 minute of application and 4.0-5.0 $\log_{10}$ after 1 minute of application (WHO, 2009).

MATERIALS AND METHODS

Modification of hand antiseptic formula

In this study, the content was modified, by making four levels of concentration ratio. The total volume was 500mL per preparation, where the ethanol content was varied in order to maintain the final alcohol level that is more than 80%. The levels of hydrogen peroxide and glycerol were also varied, while sterile water was kept constant. The following is a comparison of formulation levels between hand antiseptic with WHO-recommended formulation (2009) and hand antiseptic with modified formulation for a total volume of 500mL.

Tools

The following are tools used to make an alcohol-based hand antiseptic (10L): Jerry cans, the tank was closed to a capacity of 10L; 500mL measuring cup; 5L measuring pumpkin; and 500mL bottles.

Hand antiseptic making procedure

The following is a procedure for making hand antiseptic according to WHO recommendation (WHO, 2010), which in this case was made at the Pharmacy Department*, Fatmawati* General Hospital Jakarta:

Preparation

First, preparing the tools and materials used for the production process. The production activities were then documented. Labeling and packaging were done by making stickers/tags, inputting production date, batch number, and expiration date.

Manufacturing process (WHO, 2009)

First, the 96% alcohol solution was poured into a tightly closed container (tank/jerry can). Second, 3% H2O2 was poured using a measuring cup. Third, 98% glycerol was added using a measuring cup. Fourth, the remaining glycerol was rinsed in a clean measuring cup with sterile distilled water. Fifth, the alcohol concentration was measured using an alcohol meter with the final score more than 80%. Sixth, the tank/jerry can was covered tightly to prevent evaporation. Seventh, the liquid in the tank or jerry can was stirred for 10min so that it was evenly mixed (homogeneous) and the liquid was packed in a 500mL bottle. Finally, it was closed tightly.

Storage (WHO, 2009)

First, liquid was placed on shelves/storage cabinets. Second, it was stored for 72h at room temperature to kill spores that may grow during the formulation process. Third, the storage was labelled to inform that it is still in the process. Fourth, the alcohol concentration was measured after 72h of storage with an alcohol meter; the final alcohol level should not be less than 80%.

Raw material source

The source of raw materials in this study was from PT. Brataco Indonesia. The requirements of these raw materials are pharmaceutical grade or have guaranteed safety if it is used on humans. These raw materials have been supplemented with information on the safety data sheet (MSDS).

Testing methodology

Stage 1: Test of formulation stability rancidity testing (BPOM RI., 2008)

In the rancidity test, two 100mL tubes were used. The first tube contained 50mL of control solution (96% alcohol) while the second tube contained 50mL of hand antiseptic solution tested. Then, the odor of the control solution odor was compared to the one from the test solution. The next step is describing odor from the solution according to a scoring set. Based on the rancidity testing, a conclusion was made whether it was rancid or not rancid. Observations were made at week 1 through week 4, month 6, and month 12.

Testing for clarity/turbidity/discoloration (BPOM RI., 2008).

In the testing, two 100mL tubes were used. The first tube contained 50mL of control solution (96% alcohol) while the second tube contained 50mL of hand antiseptic solution tested. Then, they were carefully observed using a 100-watt tubular lamp (TL) for 5-10min. A comparison of each solution related to its clarity/turbidity/ discoloration was made. The result of the observations was a standard of clarity, turbidity, and discoloration. The observation was carried out at week 1 through week 4, month 6, and month 12.

Testing for final alcohol levels (WHO, 2010).

In testing the final alcohol content, two 250 mL tubes were used. The first tube contained 150 mL of control solution (96% alcohol) while the second tube contained 150 mL of hand antiseptic testing solution. Calibration was carried out to test the final level of alcohol using a calibrated alcohol meter. The alcohol control and the testing solution were poured into the first and second tubes, then the alcohol level was measured immediately after insertion of the meter. The observation was continued for 5-10 minutes by looking at the marking value on the alcohol meter, at the upper limit of the liquid surface in both control and testing solution. The solution must reach a value of more than 80%. The observation was continued at week 1 through week 4, month 6, and month 12.

Stage 2: Testing the effectiveness of antiseptics

Time-kill testing is a method of determining the effectiveness of antimicrobials with plate count techniques and analysis of percentage and log reduction (Oladosu, 2013). The procedure carried out in this test followed the standards of the ASTM (Anti-microbial Susceptibility Testing Method) E-2313. After carrying out a bacterial culture preparation, a sufficient number of test samples were placed into a sterile petri dish. Then, a number of bacterial cultures were tested (usually 1/10 or less of the volume of the test sample) by inoculating it into a petri dish beforehand and then it were immediately stirred. After determining the contact time, a small amount of bacterial mixture and testing sample was taken and put in a cup containing the nutrient agar, and then was incubated at 37°C for 24h (ASTM, 2008).

A modified formulation of 12-month hand antiseptic which was known as the most stable formulation was tested on the killing ability against bacteria. It was done to determine the effectiveness of the one-year hand antiseptic preparation. This was also done to assess the expiration period of the hand antiseptic products. At this stage of testing, hand antiseptic products with an alcohol concentration of more than 80% were used as a comparison.

The procedure for testing the killing power of the product against multi drug-resistant organism (MDRO) pathogens is as follows (ASTM., 2008): A test solution was made for the Mc Farland 0.5 equivalence in NaCl, then a 10:1 to 10:5 dilutions was carried out. Then the planting of each dilution was carried out in PCA media. 2 A sample of 4.5 cc was taken using aseptic method. Then, the control solution was made with 4.5cc aquadest (without being mixed with samples). About 500 germs were added to the sample and control solutions. Then they were homogenized with vortex. Samples and controls that had been added by germ – were taken as much as 1000µL, then they were put into a tube containing 9mL aquadest after 1min, 2min, and 5min. Planting of 1000µL into PCA was carried out each time. Incubation was carried out at a temperature of 35°C (18-24h). Calculation was made to interpret results with the following formula:

$$\% \text{ Reduction} = \frac{\text{TPC control*} - \text{TPC sample}}{\text{TPC control*}} \text{x } 99.9\%$$

* without sample

Table I. Source of Methicillin Resistance Staphylococcus aureus (MRSA). Sample number: 201908017267 (155C); Check date: August 6, 2019; Approval date: 9 August 2019; Material: Wipe the wound; Culture results: Staphylococcus aureus.

Drug	ø	S/I/R	Drug	ø	S/I/R
Penicillin class			Macrolide class		
Ampicillin (AMP)			Erythromycin E	>=8	R
Amoxycillin (AML)		R	Azithromycin (AZM)		R
Amoxyclav/Augmentin (AMC)		R	Clindamycin (DA)	>=8	R
Ampicillin Sulbactam (SAM)		R	Glycopeptide class		
Oxacillin (OX)	>=4	R	Linezolide	2	S
Cefoxitin screen	POS	+	Vancomycin (VA)	>=32	R
Benzylpenicillin	>=0.5	R	Quinolone class		
Cephalosporin class			Cifrofloxacin (CIP)	>=8	R
Cefalotin (KF)		R	Levofloxacin (LEV)	4	R
Cefazolin (KZ)		R	Moxifloxacin (MXF)	2	R
Cefuroxime (CXM)		R	Other antibiotics		
Cefoperazone (CFP)		R	Trimethoprim /Sulfamethoxazole	20	S
Ceftriaxone (CRO)		R	Tetracycline (TE)	<=1	S
Cefepime (FEP)		R	Chloramphenicol (C0		
Carbapenem class			Fosfomycin (FOS)*		S
Imipenem (IMP)		R	Tigecycline (TGC)	<=0.12	S
Meropenem (MEM)		R	Quinupristin/Daifopristin	<=0.25	S

Note: *= Kirby-Bauer method

When the result for each contact time is more than or equal to 90%, it is considered as a good result in the bacterial killing test (ASTM, 2008). MRSA was obtained from the isolation of the patient's wound swab, with an overview of the results of the resistance test as follows (Table I).

Stage 3: User Irritation Risk Test (ethical clearance number: 122/KPP/XII/2018)

User irritation risk test was carried out using a formula from the best results of the product formulation stability test. Observation of user allergic risk was conducted on volunteers who had met the inclusion criteria as follows: willing to be a subject, male or female in healthy condition, not a pregnant woman or a breastfeeding woman, aged between 18 and 65 years old. While the exclusion criteria were: Subject is pregnant or breastfeeding, has a significant medical history of a disease or dermatological condition, such as atopy, psoriasis, vitiligo, or a condition known to change the appearance of the skin or physiological response (e.g. diabetes or porphyria), medical history of a condition that will significantly affect the immune response (for example; primary immunodeficiency or acquired diseases such as HIV or AIDS; allergic diseases such as anaphylaxis, asthma, or allergic reactions due to drugs; neoplasms such as lymphoma or leukemia; rheumatoid arthritis; or systemic lupus erythematosus), medical history of significant skin cancer (e.g. melanoma or squamous cell carcinoma), and within 72h of starting as a subject using antihistamines or topical drugs on the hands. The procedure for collecting observation data using the time series method was based on two observation techniques, namely: (1) The subject is confirmed (follow-up) in the induction phase; (2) challenge phase (challenge) with general subjects as follows (FDA., 2018).

Observations on subjects confirmed in the induction phase

The subject has filled out the form; meet the inclusion criteria and are hospital employees. The induction technique is performed at five moments of washing hands with hand antiseptic: (a) before contact with the patient; (b) after contact with the patient; (c) prior to aseptic action; (d) after contact with bodily fluids; (e) after contact with the patient's environment. The induction and observation postures were carried out with a six-step hand washing technique with hand antiseptic, namely (a) on both palms, (b) behind both hands;

Table II. Scoring events Incidence of hand antiseptic allergy on the skin of the hands (FDA, 2018).

No.	Description of allergies on the skin	Score
1	No evidence of irritation	0
2	No minimal Erythema with almost no evidence of irritation	1
3	Clear Erythema and minimal edema or minimal papulae response	2
4	Erythema dan papulae	3
5	Definite edema	4
6	Erythema, edema, dan papulae	5
7	Vesicular eruption	6
8	Strong reactions spread outside the application site	7

(c) between the fingers of both hands; (d) locking the fingers; (e) fingertips and nails of both hands. Induction of treatment was carried out in a normal work cycle, with a duration of observation: 70h, i.e. 7h/shift times ten (10) working days. Induction of treatment was carried out in six steps of hand washing with a five-time hand antiseptic with 2-5mL MD4 hand antiseptic product, with a minimum contact duration of 24s to 60s. The report confirmed if any irritation/allergy was done daily during the span of observation either by the subject or by the researcher. If there are reports of irritation/allergy, it is advisable to consult a dermatologist (genital dermatologist); and researchers provide an assessment based on the results of the assessment, with the following criteria.

Termination of the subject was carried out if there was an irritation/allergy due to the treatment of hand antiseptic-MK4 in induction phase; and did treatment based on clinical pathways (CP) and clinical practice guidelines (PPK) that applied in hospitals (Table II).

Observation in challenge phase (challenge) with general subjects (FDA, 2018).

Subjects who were willing to volunteer should meet the inclusion criteria. The induction technique is performed at five moments of washing hands with hand antiseptic: (a) before contact with the patient; (b) after contact with the patient; (c) prior to aseptic action; (d) after contact with bodily fluids; (e) after contact with the environment. The induction and observation postures were carried out with a six-step hand washing technique with hand antiseptic, namely (a) on both palms; (b) behind both hands; (c) between the fingers of both hands; (d) locking the fingers; (e) fingertips and nails of both hands. Induction of treatment was carried out by one-handed hygiene practice, observing only the hospital environment. Induction treatment: perform a six-step hand washing with hand antiseptic; with 2-5mL of MK.4-hand antiseptic product, with a minimum contact duration of 24s to 60 s. Reports confirmed if there was irritation/allergy after washing hands with hand antiseptic, either by the subject or by the researcher. If there are reports of incidents of irritation/allergy, it is advisable to consult a dermatologist (genital dermatologist); and researchers provide an assessment based on the results of the assessment, with the criteria as mentioned above.

RESULT AND DISCUSSION

Stage 1: Result and discussion of formulation test

The testing period was conducted from January 2019 - December 2019 or for 12 months. Testing was done by comparing five formulation models. Formulation 1 was the WHO-recommended formula, while formulation 1 - 4 were modifications on the concentrations of active substances (Table III). Observation results on the WHO-recommended formula showed a peculiar rancid odor in week I storage until the 12th month. In MK.1, the rancid odor was identified in week III. In MK.2, it was identified in week IV, while in MK.3, rancidity was identified in the 6th month. Whereas in MK.4, no rancid odor was found until the storage process in the 12th month. The statements "odorless", "practically odorless", "characteristic odor is weak" or otherwise, were determined by observation after the material had been exposed to air for 15min after the container of hand antiseptic solution was opened. The rancid odor mentioned is only descriptive of the material concerned (BPOM. RI., 2008).

Table III. Results of formulation test

Criteria	Observation result						Observation Parameter
	week I	week II	week III	week IV	month VI	month XII	
Formula standard WHO:	+	++	+++	+++	+++	+++	Rancid odor
	clear	clear	clear	clear	clear	Clear	Clarity
Batch:	no	no	no	no	no	no	Color change
HR-ORG/01-2019/1/2/3/4	80%	80%	80%	80%	80%	80%	Alcohol concentration
concentration modification 1	Neg.	Neg.	+	+	++	++	Rancid odor
	clear	Clear	clear	clear	clear	Clear	Clarity
Batch:	no	no	no	no	no	no	Color change
HR-MD1/01-2019/1/2/3/4	85%	85%	84%	83%	83%	83%	Alcohol concentration
concentration modification 2	Neg.	Neg.	Neg.	+	+	+	Rancid odor
	clear	Clear	clear	clear	clear	Clear	clarity
Batch:	no	no	no	no	no	no	Color change
HR-MD2/01-2019/1/2/3/4	86%	86%	84%	83%	83%	83%	Alcohol concentration
concentration modification 3	Neg.	Neg.	Neg.	Neg.	+	+	Rancid odor
	clear	Clear	clear	clear	clear	Clear	Clarity
Batch:	no	no	no	no	no	no	Color change
HR-MD3/01-2019/1/2/3/4	87%	85%	84%	84%	84%	84%	Alcohol concentration
concentration modification 4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Rancid odor
	clear	Clear	clear	clear	clear	Clear	clarity
Batch:	no	no	no	no	no	no	Color change
HR-MD4/01-2019/1/2/3/4	87%	87%	87%	87%	86%	86%	Alcohol concentration

Notes: M.I;II;III;IV = week 1-4 ; (-) = Negative ; (+) = slightly positive ; (++)= medium positive; (+++) = absolutely positive

Table IV. Percentage of bacterial killing test results on formula MK.4

Kind of bacteria	Time of Contact*				% reduction of colony numbers
	Without sample/control in *Colony Forming Unit* (CFU)		With sample in *Colony Forming Unit* (CFU)		
methicillin-resistant *staphylococcus aureus* (MRSA)	1Min	38	1Min	0 (null)	99,9%
	2Min	51	2Min	0 (null)	99,9%
	5Min	52	5Min	0 (null)	99,9%

The appearance of this particular rancid odor is probably due to the presence of glycerin (glycerol) in hydrolyzed alcohol. Glycerol in this formulation functions as an emollient to prevent irritation to the skin due to alcohol ingredients. Glycerol according to its molecular formula, $C_3H_8O_3$, is a simple polyol compound. It is a colorless, odorless, and thick liquid that tastes sweet and non-toxic (Christoph and Ralf, *et al*., 2006). Glycerol can change into Reuterin (3-hydroxypropionaldehyde), which is an organic compound with the formula of $HOCH_2CH_2CHO$. It is a bi-functional molecule containing hydroxyl and aldehyde functional groups. Whereas aldehydes have various properties and it depends on the rest of the molecule. Smaller aldehydes are more soluble in water, formaldehyde, and acetaldehyde. Aldehydes are volatile and have a pungent odor (Katarzyna, *et al*., 2011).

Based on observation of the final alcohol concentration (Table III), the WHO-recommended formula did not experience a decrease in concentration. It remained at 80% after 12 months of storage. In MK.1 and MK. 2, alcohol concentration decreased from week 3 from 84% to 83% at 12 months of storage. In MK.3, alcohol concentration decreased in week 2 from 85% to 84% at 12 months of storage. In MK.4, alcohol concentration decreased in week 4 from 87% to 86% at 12 months of storage. In general, all formulations met the standards in terms of concentration required by the WHO. The final alcohol concentration of hand antiseptic preparation was more than 80% (WHO., 2010).

Table V. Percentage of bacterial killing test results on Softaman

Kind of bacteria	Time of Contact*				% reduction of colony numbers
	Without sample/control in *Colony Forming Unit* (CFU)		With sample in *Colony Forming Unit* (CFU)		
methicillin-resistant *staphylococcus aureus* (MRSA)	1Min	51	1Min	0 (null)	99,9%
	2Min	52	2Min	0 (null)	99,9%
	5Min	58	5Min	0 (null)	99,9%

However, several studies have shown that 60-70% ethanol can lyse bacteria including hepatitis B virus (HBV) and it probably can also lyse hepatitis C virus. In a porcine tissue carrier model used to study antiseptic activity, it was found that 70% ethanol and 70% isopropanol can reduce the titer of enveloped bacteriophages more effectively than antibacterial soap containing 4% CHG. Ethanol has bacterial or enveloped (lipophilic) activity such as herpes simplex virus (HSV), HIV, influenza virus, RSV, and vaccine virus, which are generally sensitive to alcohol when tested *in vitro* (WHO, 2009).

Stage 2: Percentage of bacterial killing test results

The bacterial killing test was done for MK.4 which was selected from the formulation test. This formulation had the best relative results compared to other formulation with various modifications of concentration.

The testing period was carried out after the formulation was kept for 12 months or one year. This was done at the same time by testing the effective age of the sample and testing its effectiveness in terms of pathogen reduction in percentage (%) against bacteria that are known to have resistance, in this case, methicillin-resistant staphylococcus aureus (MRSA). *Staphylococcus aureus*, gram-positive bacteria, pathogens with coagulase-positive originating from the Staphylococcaceae family, are spherical bacteria with diameters close to 1µm resembling grape clusters. *S. aureus* is a commensal that often appears without symptoms on parts of the human body such as skin, skin glands, and mucous membranes, including the healthy nose and intestines of humans (Gould, and Chamberlaine, 1995). Studies show that about 20% of individuals are persistent carriers of *S. aureus* and about 30% of individuals as intermittent carriers, while the other 50% of individuals are not carriers (Wertheim, *et al*., 2005). Therefore, this colonization significantly increases the chance of infection by providing a reservoir of pathogens. In most cases, individuals infected with *S. aureus* strains are usually carried as commensal (Katayama, *et al*., 2000).

In this study, the source of MRSA was obtained from the results of the wound swab isolation, from the laboratory of Fatmawati General Hospital. The test was conducted at the microbiology laboratory of the University of Indonesia (UI), with the following results. Sample without active substances, at the first minute, the number of colonies was 38 CFU (Table IV). At the second minute, it was 51 CFU, and at the 5th minute, it was 52 CFU. When it was compared to the results from hand antiseptic MK.4, it is known that at the 1st, 2nd, and 5th minute, it was 0 CFU. Based on the calculation on concentration reduction in the 1st, 2nd, and 3rd minute, the bacterial killing percentage was 99.9%. This shows that the quality of the hand antiseptic product was good because based on the results of the bacterial killing test, an antiseptic is declared good if the percentage obtained is more than or equal to 90% for each contact time.

As a comparison, a manufacturer hand antiseptic was also tested, namely Softaman. The selection of this product is due to having an alcohol content of more than 80% as WHO has suggested. Sample without active substances was tested at the first minute. The number of colonies was 51 CFU (Table V). At the second minute, it was 55 CFU and at the 5th minute, it was 58 CFU. When it was compared to the results of the test with the active substance of Softaman hand antiseptic, it is known that at the 1st, 2nd, and 5th minute, the total was 0 CFU. Based on the calculation, the colony reduction at the 1st, 2nd, and 3rd minute was 99.9%. This shows that the quality of the hand antiseptic product was good, because based on the results of the bacterial killing test, an antiseptic is declared good if the percentage obtained is more than or equal to 90% for each contact time (ASTM, 2008).

Table VI. Analysis of subject demographic data

Group	Status	Number of subjects	Percentage
Profession	Nurse	6	26.1
	Caregiver professional	11	47.8
	miscellaneous	6	26.1
	Total	23	100
Age	17-25 y.o	1	4.3
	26-35 y.o	8	34.8
	36-45 y.o	9	39.1
	46-55 y.o	4	17.4
	56-65 y.o	1	4.3
	Total	23	100.0
Gender	Male	7	30.4
	Female	16	69.6
	Total	23	100
Location	Inpatient room- *Teratai*	2	8.7
	Inpatient room -*Anggrek*	7	30.4
	Inpatient room -GPS	3	13.0
	Emergency Unit	1	4.3
	Pharmacy	10	43.5
	Total	23	100.0
Allergic	Yes	4	17.4
History	None	19	82.6
	Total	23	100
User Group	Employee	16	69.6
	Public	7	30.4
	Total	23	100

The bacterial killing test was conducted on MRSA to assess the ability of hand antiseptic in reducing colony of microbes. This is also due to infection of MRSA strains resulting in higher mortality than infections caused by species that are susceptible to methicillin. This results in longer hospital stays and increased health care costs (Fortuin, *et al*., 2015). MRSA strains produce changes in penicillin-binding protein associated with decreased affinity for most semisynthetic penicillin. The protein is encoded by the genes obtained, namely mecA (Lakhundi, and Zhang, 2018). This gene is resistant to the methicillin-resistant component of the cellular genetic element (MGE) which is characterized by the acquisition of Staphylococcal Cassette Chromosome mec (SCCmec) insertion of genetic elements that move into chromosomes from susceptible strains. The acquisition of antimicrobial resistance is causing new challenges for the medical world in terms of treatment and control of staphylococcal infections. MRSA in many cases accounts for 25 to 50% of *S. aureus* infections in hospitals (Lakhundi, and Zhang, 2018). This infection is a major concern because of their high morbidity and mortality and resistance to penicillin and other lactam antibiotics, except ceftaroline and ceftobiprole.

Stage 3. User irritation risk test

This user irritation risk test was conducted on 23 subjects divided into 11 subjects in follow up-induction scheme and 12 subjects in the challenge scheme.

Based on demographic data (Table VI), there were 4 subjects or 17.4% who had a history of allergic reaction confirmed since the first day, and 19 subjects or 82.6% with no allergic history based on subject recognition. In the follow-up (induction) subject group, there were 110 treatments, while in the challenge subject group 12 treatments were carried out. In the induction-follow-up group, there were 11 selected subjects and their adherence in using hand antiseptic at five moments was constantly followed/observed.

Table VII. Number of subject and treatment in each subject group

GROUP	PHASE	Number of subjects	Number of treatments
Subject group	Induction follow-up	11	110
	challenge	12	12
	Total	23	122

Table VIII Monitoring time series exposure on *Induction-Follow-up* group

Code	Groups	Incident irritation/ allergic	Monitoring Time Series (Table 1)									
			Day1		Day2		Day3		Day4		Day5	
			Σ pap.	Score	Σ pap.	Score	Σ pap.	Score	Σ pap.	Score	Σ pap.	Score
F-01	FI	None	21	0	21	0	26	0	26	0	24	0
F-02	FI	None	24	0	22	0	29	0	22	0	28	0
F-03	FI	None	29	0	21	0	23	0	26	0	24	0
F-04	FI	None	25	0	26	0	27	0	26	0	20	0
F-05	FI	None	21	0	21	0	29	0	24	0	24	0
F-06	FI	None	23	0	22	0	25	0	26	0	24	0
F-07	FI	None	19	0	18	0	21	0	20	0	20	0
F-08	FI	None	19	0	18	0	23	0	22	0	16	0
F-09	FI	None	23	0	22	0	19	0	18	0	14	0
F-10	FI	None	22	0	20	0	25	0	18	0	16	0
F-11	FI	None	21	0	18	0	21	0	22	0	20	0
Average exposure			22		21		24		23		21	
Code	Groups	Incident irritation/ allergic	Monitoring Time Series (Table 2)									
			Day6		Day7		Day8		Day9		Day10	
			Σ pap.	Score	Σ pap.	Score	Σ pap.	Score	Σ pap.	Score	Σ pap.	Score
F-01	FI	None	21	0	21	0	26	0	26	0	24	0
F-02	FI	None	24	0	22	0	29	0	22	0	28	0
F-03	FI	None	21	0	21	0	26	0	26	0	24	0
F-04	FI	None	25	0	26	0	27	0	26	0	20	0
F-05	FI	None	22	0	21	0	29	0	24	0	24	0
F-06	FI	None	23	0	22	0	25	0	26	0	22	0
F-07	FI	None	19	0	16	0	19	0	20	0	20	0
F-08	FI	None	17	0	18	0	19	0	22	0	18	0
F-09	FI	None	19	0	20	0	19	0	23	0	20	0
F-10	FI	None	17	0	29	0	21	0	20	0	16	0
F-11	FI	None	19	0	18	0	19	0	20	0	20	0
Average exposure			21		21		24		23		21	

Information:FI = Follow up-Induction ; Σ pap.= number of exposure;

Observations were made for 10 working days, divided into two periods, namely: the first week of observation on Monday and the second week of observation, which was on Monday until Friday. So, there was an interval between two observations for 2 days, Saturday and Sunday. Observations were only conducted during working hours or morning service shifts (8.00 AM - 3.00 PM).

During the observation period, user irritation risk was scored using the following scoring values. Determination of irritation risk grade was done collaboratively with a dermatologist. Based on the observational data series, it is known that during 10 days of observation of 11 subjects, a total of 2,435 data were recorded. With an average value per day, there were 244 exposures from the induction follow-up group, where each subject on average got 22 exposures.

On the 5th day of observation, subject of code F-01 run an irritation risk. After confirmation by a dermatologist, the subject experienced minimum Erythema that was barely visible or in a score of 1, with a 30-minute phasing procedure (Table VIII).

Table IX. Monitoring Time Series exposure on the challenge group

Subject	Groups	Profession	Age	Sex	Allergy historical	Group user	Incident irritation/allergic	*Monitoring Time Series* Nul.Day	
								Σ pap.	Score
C-01	CT	PPA	26-35	L	None	Employees	None	1	0
C-02	CT	PPA	26-36	P	Yes	Employees	None	1	0
C-03	CT	PPA	36-45	P	None	Employees	None	1	0
C-04	CT	PPA	36-45	P	Yes	Employees	None	1	0
C-05	CT	PPA	26-36	L	None	Public	None	1	0
C-06	CT	Other	36-45	P	None	Public	None	1	0
C-07	CT	Other	36-45	P	None	Public	None	1	0
C-08	CT	Other	56-65	P	None	Public	None	1	0
C-09	CT	Other	26-35	L	None	Public	None	1	0
C-10	CT	Other	17-25	L	None	Public	None	1	0
C-11	CT	Other	46-55	P	None	Public	None	1	0
C-12	CT	PPA	36-45	P	Yes	employees	None	1	0

PPA = professional *Pemberi Asuhan* ; CT = Challenge – *Tantangan*

Table X. Test data for the significance of irritation/allergic risk vs. number of exposure on the use of hand antiseptic MK.4

		Total exposure on the use of *Hand antiseptic* Null day 1-10	**Total exposure on the use of *Hand antiseptic* day 1- 10**	
Allergic or irritation risk	Pearson Correlation	1.000**	1.000**	1.000**
	Sig. (2-tailed)	.000	.000	.000
	N -*exposure*	122	122	122

**. Correlation is significant at the 0.01 level (2-tailed).

The risk could be self-limiting and the subject could continue until the end of observation.

Based on demographic data, subject with code F-01 had an allergic history. In the challenge phase, an open scheme was made where the chosen subject only got one exposure. Observation of the irritation risk was done before hand was cleaned with hand antiseptic up to 30-60min after use. The next data was in the challenge phase. There were 3 subjects who had an allergic risk, but based on the results of observation, there was no irritation risk in general.

Statistical analysis (T-test) and correlation between the "exposure amount" based on time series data vs. irritation/allergic event data, it is known as follows (Table IX). Pearson correlation value was 1, so there is a strong relationship between each variable. Based on the value of sig. between variables, it is known that the alpha value is equal to 0.00. It means that H0 is accepted. In general, hand antiseptic MK.4 in this study does not have the risk of causing irritation/hypersensitivity (Table X).

CONCLUSION

Hand antiseptic products show good results: In formulation, MK.4 shows the best properties compared to other formulations. Based on the bacterial killing test results, the percentage of germ reduction for each contact time was more than or equal to 90%. In general, the formulation used did not pose a significant risk of irritation to the user.

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