EFFECTS OF ETHANOL-BASED ANTISEPTIC SOLUTIONS ON GRAM-NEGATIVE BACTERIA

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ABSTRACT

Introduction: *Gram-negative bacteria infections cause diseases, namely skin infection until sepsis, including nosocomial infection. Prevention by antiseptic application is the way to inhibit infection. Some antiseptic compounds that have been used show resistance according to some reports.* ***Aim:*** *Determine the effects of ethanol-based antiseptic solutions against Gram-negative bacteria.* ***Methods:*** *Discs saturated with ethanol-based antiseptic solutions were affixed to Muller Hinton agar smeared by Gram-negative bacteria such as Acinetobacter baumannii ATCC BAA-747, Escherichia coli ATCC 25922, Klebsiella pneumonia ATCC BAA-1706, Proteus vulgaris ATCC 6380, Pseudomonas aeruginosa ATCC 27853, and Salmonella sp. The diameter of the inhibition zone was read after 24 hours. Solutions are considered sensitive if the inhibition zone of growth diameter is more than 6 millimeters (Kirby-Bauer disc diffusion method).* ***Result:*** *Acinetobacter baumannii and Salmonella sp are sensitive to a solution consisting of ethanol 80 % and ethanol 80 %+ H2O2 0.15%. Pseudomonas aeruginosa and Escherichia coli are sensitive only to a solution consisting of ethanol 80 %+ H2O2 0,15%. As for Klebsiella pneumonia and Proteus vulgaris, they are resistant to both solutions.* ***Conclusion****: The use of ethanol-based antiseptic solutions with or without H2O2 0.15% addition does not effectively eliminate all gram-negative bacteria from the surface. The addition of 0.15% H2O2 to the antiseptic solution showed a better barrier effect than the solution containing only 80% ethanol. Adding other additives needs to be investigated further to formulate a better antiseptic solution against Gram-negative bacteria.*

Keywords: *Ethanol, Antiseptic, Gram-Negative Bacteria*

ABSTRAK

Latar belakang : Infeksi bakteri Gram negatif menimbulkan penyakit, mulai dari infeksi kulit hingga sepsis, termasuk di antaranya adalah infeksi nosokomial. Penggunaan antiseptik merupakan salah satu cara untuk menghambat infeksi. Berbagai laporan menunjukkan adanya resistensi dari beberapa senyawa antiseptik yang sering digunakan. **Tujuan**: Menentukan efek larutan antiseptik berbasis etanol terhadap bakteri Gram negatif **Metode**: Cakram jenuh dengan larutan antiseptik berbasis etanol diletakkan pada agar Muller Hinton yang mengandung

bakteri Gram negatif, antara lain *Acinetobacter baumannii* ATCC BAA-747, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC BAA-1706, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 27853, dan *Salmonella sp*. Diameter zona hambat diukur setelah 24 jam. Larutan dianggap sensitif jika diameter zona hambat lebih dari 6 milimeter (metode difusi cakram Kirby-Bauer). **Hasil**: *Acinetobacter baumannii* dan *Salmonella sp* sensitif terhadap larutan yang terdiri dari etanol 80% dan etanol 80% + H2O2 0,15%. *Pseudomonas aeruginosa* dan *Escherichia coli* sensitif hanya pada larutan yang terdiri dari etanol 80% + H2O2 0,15%. Sedangkan untuk *Klebsiella pneumonia* dan *Proteus vulgaris* resisten terhadap kedua larutan tersebut. **Kesimpula**n: Penggunaan larutan antiseptik berbasis etanol dengan atau tanpa penambahan H2O2 0,15% tidak efektif mengeliminasi semua bakteri Gram negatif dari permukaan. Penambahan 0,15% H2O2 pada larutan antiseptik menunjukkan efek menghambat pertumbuhan yang lebih baik dibandingkan dengan larutan yang hanya mengandung etanol 80%. Penambahan zat aditif lain perlu diteliti lebih lanjut untuk menyusun formula larutan antiseptik yang lebih baik terhadap bakteri Gram-negatif.

Kata Kunci: Etanol, Antiseptik, Bakteri Gram-Negatif

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INTRODUCTION

Gram-negative bacteria are considered cosmopolitan microbes, whose ability to infect humans represents a huge and emerging threat to public health and a burden on health and economy. They cause significant morbidity and mortality worldwide, both in communities and hospitals, especially in immuno-compromised individuals. These microorganisms colonize parts of the body such as the respiratory tract, digestive tract, and skin that would accommodate the spread to other parts of the host's body. Nosocomial infections, particularly caused by Gram-negative bacterias, have shown resistance from antibiotics that challenged health care professionals (1,2).

Gram-negative bacteria are divided into *Enterobacteriaceae,* which are common in the intestine, non-fermenters bacteria, and other Gram-negative bacteria. Among the *Enterobacteriaceae* group are *Escherichia coli, Salmonella sp., Klebsiella sp*. and *Proteus sp*. Gram-negative non-fermenters group are also called Gram-negative bacillus, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Chlamydia trachomatis, Haemophilus spp., Helicobacter pylori,* and *Neisseria* are

classified in other Gram-negative bacteria (2).

Enterobacteriaceae is the largest group among all gram-negative bacteria that occur in nature, with a percentage of almost 80%. The disease can be fatal if not treated properly. *Enterobacteriaceae*-infected systems such as the urinary tract, respiratory tract, intestine, and central nervous system can lead to complications that are difficult to treat, such as sepsis, endotoxic shock, and death. *Proteus* species also have been linked to Crohn's disease, a chronic inflammatory bowel disease that affects the digestive tract. (4). On the other hand, although the incidence of infection due to While Gram-negative bacilli is lower than that of Enterobacteriaceae, they account for most cases of hospital-related complications such as ventilator associated pneumonia and catheter-related bloodstream infections (1,2).

There are three layers in the envelope structure of Gram-negative bacteria. The outermost membrane is the key structure that distinguishes it from Gram-positive bacteria and consists of phospholipids and lipopolysaccharides. The second layer acts as a cell skeleton in the form of a disaccharide N-acetyl glucosamine-N-acetylmuramic acid chain as the phospholipid bilayer is the last layer of the membrane (1).

Prevention with antiseptics is one strategy to fight and reduce the risks of transmission. The World Health Organization (WHO) recommends the use of antiseptics, especially for medical personnel. Keeping hands clean from contamination of bacteria and other pathogenic microbes isan important step in avoiding human-to-human or surface-to-human transmission (6,7).

Alcohol, including ethanol, isopropanol or isopropyl alcohol, and n-propanol, have been globally used as topical antiseptics (skin) since the 1800s. Compared to washing hands with soap or other antiseptic agents, rubbing hands with alcohol-based antiseptics is more time-saving, easier, effective in eliminating pathogenic microorganisms, and less irritating to the skin (8). WHO recommends the alcohol content for antiseptic is 60-90%, while the recommended concentration by the Ministry of Health of the Republic of Indonesia ranges from 70-80% (6,9).

The antimicrobial activity of alcohol may be related to protein denaturation. Another theory proposed is to directly influence the ribosome and RNA polymerase that inhibits protein and mRNA synthesis. As a result, some of the vital metabolic functions of cells are disrupted, loss of cell integrity, and damage to cell membranes, resulting in cell death (7,10).

Therefore, the main problem at this time is understanding whether the antiseptic

regimens currently available are able to meet the standards of eliminating pathogenic microorganisms, especially Gram-negative bacteria. Several studies have reported resistance or decreased effectiveness of antiseptic agents, such as alcohol, chlorhexidine, quaternary ammonium compounds, triclosan (6,7,11). Due to these reports, this study is aimed at the efficacy of the commonly used ethanol-based antiseptics against Gram-negative bacteria.

METHODS AND MATERIAL

The materials and tools used in this in vitro experimental research are ethanol, glycerin, H2O2, aquabidest, measuring cup, glass stirrer, alcoholmeter, glass pipette, glass bottle, MacConkey agar media, and Muller Hinton agar media. Antiseptic solutions that were tested consisted of solutions consisting of ethanol 80% v / v, glycerin 8% v / v, water (add 100% v / v) and solutions consisting of ethanol 80% v / v, H2O2 0.15% v / v, glycerin 8% v / v, water (add 100% v / v). Concentrations are given as either percentage of volume (= ml / 100 ml, abbreviated% v / v). All the solutions were prepared as previously described in WHO guidelines (6).

The alcohol content in the antiseptic solution is determined using an alcoholmeter. The alcoholmeter was immersed while rotating in 200 mL antiseptic solution. Alcoholmeter is allowed to spin until it stops. The alcohol content is indicated by a number that appears on the upper surface limit of the solution.

The Gram-negative bacteria used were *Acinetobacter baumannii* ATCC BAA-747, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC BAA-1706, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella sp.* All bacteria were obtained from the Central Health Laboratory Ministry of Health of the Republic of Indonesia.

Susceptibility test

In this study, to test the sensitivity of bacteria to antiseptic solutions, a disc diffusion assay method was used, which was developed by W. Kirby and A. Bauer as previously described (12). Each bacteria were cultured in MacConkey agar for 24 hours. The inoculum was made at a concentration equivalent to a 0.5 McFarland standard. The prepared bacterial suspension was then inoculated on a plate of Muller Hinton agar and paper disc impregnated with an antiseptic solution (test material), and antibiotics as control were added to the plate. The bacteria are allowed to grow overnight; after that, the zone of inhibition diameters was measured to the nearest millimeter, including the diameter of the disc.

As for control, we used gentamycin

Ten mcg for *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa,* and ciprofloxacin five mcg for *Salmonella sp*. The presence or absence of an inhibition zone around the disc determines the bacteria's sensitivity to each antiseptic solution. The diameter of the zone of inhibition more than 6 mm is interpreted as sensitive and resistant when the measured diameter is 6 mm or less.

The study was carried out at the Center for Health Laboratory of the Ministry of Health of the Republic of Indonesia, 18th Karang Menjangan Street, Surabaya, East Java, Indonesia. All data were analyzed descriptively.

RESULT

After being incubated for 24 hours, the test material for each solution was observed and measured. The zone of inhibition of growth diameter was measured by observing the sharply marginated circle of bacterial growth around the disk, as shown in Table 1.

Table 1. Zone Of Inhibition Of Growth From Disc Diffusion Test

Gram-negative bacteria	Ethanol 80%	Ethanol 80% + H2O2 0.15%
Acinetobacter baumannii. ATCC BAA-747	Sensitive (8 mm)	Sensitive (18 mm)
Pseudomonas aeruginosa. ATCC 27853	Resistance (≤ 6 mm)	Sensitive (10 mm)
Klebsiella pneumonia. ATCC BAA-1706	Resistance (≤ 6 mm)	Resistance (≤ 6 mm)
Escherichia coli. ATCC 25922	Resistance (≤ 6 mm)	Sensitive (7 mm)
Salmonella sp.	Sensitive (8 mm)	Sensitive (9 mm)
Proteus vulgaris. ATCC 6380	Resistance (≤ 6 mm)	Resistance (≤ 6 mm)

The assay results on *Acinetobacter baumannii* and *Salmonella sp*. showed the inhibition zone for both solutions was more than 6 mm, which indicated that the antiseptic solution could inhibit bacterial growth. *Pseudomonas aeruginosa* and *Escherichia coli* are sensitive only to a solution consisting of ethanol 80 %+ H2O2 0,15% with diameters of the zone of inhibition is 10 mm and 7 mm. As for *Klebsiella pneumonia* and *Proteus vulgaris,* they are resistant to both solution

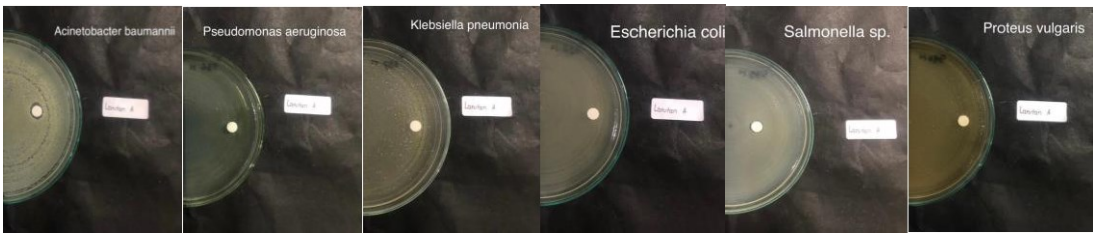

Figure 1. Disc diffusion assay of ethanol 80% antiseptic solution

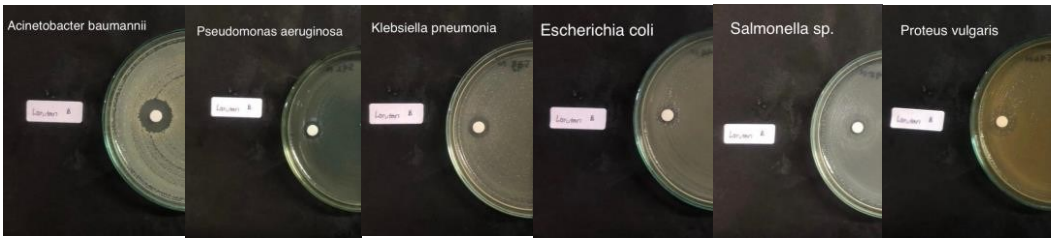

Figure 2. Disc diffusion assay of ethanol 80% + H2O2 0.15% antiseptic solution

DISCUSSION

Excessive use of topical antiseptics is suspected to be one of the causes of the increasing number of multidrug- resistant pathogens that the medical world has to face. Ethanol-based antiseptics are currently the most widely used substances, and regulation of topical antiseptic applications has received less attention than antibiotics leading to concern about the development of antiseptics resistance (13).

This study used antiseptic solutions contains 80% ethanol as the main compound as recommended range concentrations by WHO and the Ministry of Health of the Republic of Indonesia; also antiseptic containing a total amount of up to 70% alcohol is significantly less effective than at least 80 %. Absolute alcohol does not show any better bactericidal properties than,diluted alcohol (14,15).

Solution with 80% ethanol as the main component of antiseptic only effective for *A. baumanni* and *Salmonella sp*. The combination with 0.15% H2O2 has been shown to increase the inhibitory,ability of antiseptics solutions in this study against *P. aeruginosa* and *E. coli*.

Hydrogen peroxide (H2O2) is associated primarily with its oxidation activity. H2O2 can cause damage to various cellular processes, impaired protein synthesis, and loss of integrity and cellular homeostasis. The mechanism of action is through the ability of trace metals it contains to catalyze the formation of hydroxyl radicals which lead to the breaking of the nucleic acid chains of DNA, protein backbones, and cell membranes disruption (7,15). Alcohol cannot eliminate fungal spores, which is the main objective WHO recommends adding H2O2 to alcohol-based antiseptic formula. Combining chemicals in an antiseptic are better at preventing microbial resistance

than an antiseptic with a single chemical (6,16).

Ethanol-based antiseptics are considered effective but easily evaporated. This characteristic causes its bactericidal ability does not last long; also alcohol does not equip any residual antimicrobial activity. Adding other ingredients like,H202 and emollients (glycerin) decrease the evaporation rate, thus extending the time of antimicrobial activity. In addition, emollients are also aimed at reducing the irritating side effects often associated with the frequent use of alcohol (7,11).

Gram-negative bacteria's outer membrane provides an effective barrier to antiseptics, which shows the results of sensitivities are significantly different from Gram-positive bacteria. For example, the outer membrane of *P. aeruginosa* has notable differences in LPS composition and in the cation content of the outer membrane that is responsible for its high resistance. The high Mg^{2+} content results in a strong bond between the lipopolysaccharide and their small size, inhibiting the diffusion process across the membrane. Another tolerance mechanism report are associated with the upregulation of efflux mechanisms and changes in the membrane lipid composition, namely the increase in the amount of long-chain fatty acids of the cell membrane. Therefore, the hydrophobic nature increases, making it difficult for the biocide to penetrate through the membrane (2,7).

Things that need attention are in this present study, *K. pneumoniae* and *P. vulgaris*, they are resistant to both solutions which shows that the ethanol-based antiseptics commonly used in daily life are not guaranteed to be effective in preventing the spread of pathogens. These bacterias belong to the *Enterobacteriaceae* group with a character capable of producing Catalase (17). Catalase has a protective function from oxidative cell damage due to reactive oxygen species (ROS), in this case, external hydrogen peroxide contained in antiseptics. Hydrogen peroxide is broken down into oxygen and water. This process is catalyzed by the KatA catalase enzyme produced by certain Catalase-positive bacterias, resulting in increased tolerance to antiseptic solutions (15).

CONCLUSION

Certain Gram-negative bacteria like *K. pneumonia* and *P. vulgaris* are resistant to ethanol-based antiseptics commonly used in daily basics. The addition of 0.15% H2O2 to the antiseptic solution showed a better barrier effect than the solution containing only 80% ethanol. There is a necessity to conduct trials combining with other compounds to make a more effective antiseptic formula. Regular surveillance

should be done to detect any incidence of resistance to antiseptics in order to prevent the further transmission of pathogenic microorganisms.

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