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The Effect of Low Power Ultrasonic Wave Exposure to Suppress Methicillin-Resistant *Staphylococcus aureus* (MRSA) *In Vitro*

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ABSTRACT

The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection keeps increasing in every part of the world. Currently, the infection prevalence of MRSA has reached 70% in Asia. In Indonesia in 2006 the prevalence was 23.5%; the infection prevalence of MRSA in RS Atmajaya Jakarta reached 47%, in RSUP Dr. Moh. Husin Palembang reached 46%, and RSUD Abdul Moeloek Lampung in 2013 reached 38.4%. MRSA is multiresistant to antibiotics and is hard to kill compared to most other negative gram bacteria. The purpose of this research is to find the lethal power and exposure of ultrasonic waves to kill MRSA, monitoring its effects via changes in shape, size, structure and Gram staining as indicators. The observations were done macroscopically by culturing the MRSA in a petri dish filled with Chromagar MRSA medium, while the morphological observations of MRSA were done by SEM, changes in the structure using TEM, and changes in the color of MRSA cells using Gram staining. Ultrasonic wave exposure, at a lethal power = 8.432 watt, killed a significant percentage of MRSA over the control (p = 0.000). The death indicators of the MRSA due to exposure to ultrasonic waves of various power were: changes in shape of MRSA affected by ultrasonic power (p = 0.005), changes in size is not affected by ultrasonic power (p= 0.470), the stain of MRSA cell staining from purple to pink affected by ultrasonic power (p = 0.000), all compared with the control. MRSA died due to necrosis, with physical evidence of the MRSA death such as mechanical stress marked by swollen MRSA cell, shift cell wall, crack and tears, cavitation marked by pieces of MRSA cell in the field of view due to explosions inside the cell, change to an irregular cell shape, and changes in color from black to transparent.

Keywords: *Cavitation, mechanical stress, MRSA, SEM, TEM, ultrasonic*

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has developed from selection pressures caused by inappropriate antibiotic therapy exposure [1]. MRSA is multiresistant to many antibiotics used to control negative Gram bacteria [2]. Thus, MRSA is one of the most prominent pathogens causing health problems in the human community as well as livestock [3, 4, 5, 6, 7]. MRSA must be eradicated by effective methods that will not cause further resistance to develop.

Ultrasonic waves are sound waves in which the frequency is not more than 20 kHz. The non-thermal effect of these waves is cavitation, the formation of gas bubbles, unidirectional flow, and stable mechanic power which touches all of the surface [8]. Ultrasonic waves with lower frequencies have a longer wavelength and a larger amplitude to input the energy supplied, causing larger disruptions in the medium and increasing molecular movement [9]. Generally, when exposing cells to ultrasonic waves, cavitation occurs using ultrasonic

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waves with low frequency and are used to break cell membranes and make the cells lyse [10 – 16]. Cavitation produced by ultrasonic waves can break bacterial cell walls, as well as structural and functional components via intracellular cavitation [17, 18]. Ultrasonic energy absorption by enzymatic proteins can cause changes in enzymatic activity. The hypothesis of frequency resonance explains two biological mechanisms that might change protein function as the result of ultrasonic energy absorption [19].

When bacteria are in intensive fields of ultrasonic waves with high frequency, then the bacteria will suffer great vibratory disruptions and a large voltage, in which sealing and stretching occur because of differences of pressure between the inside and outside of the cell wall. When the strain in the cell wall is large enough to exceed the limit of elasticity then the cell wall will be torn and the bacteria will die. Another mechanism of interaction of ultrasonic waves with bacteria is the effect of cavitation. The effect of cavitation can break molecular bonds. The broken H_2O molecule forms H^+, OH^-, and HO_2 radicals as well as a great deal of H_2O_2, which will oxidize organic molecules throughout the bacterial cell causing cell death [20].

Various experiments have studied controlling *Escherichia coli* bacteria using ultrasonic waves. Dehghani and Hadi (2005) used 42 kHz frequency, 70-watt power waves and were able to kill 99.80% of the bacteria [21]. Herceg et al. (2013) used 20 kHz frequency, 600-watt power waves, which combined with high temperatures, were able to kill *E. coli* 3014, *S. aureus* 3048, *Salmonella* sp. 3046, *Listeria monosytogeneses* ATCC 23074, and *Bacillus cereus* 30 [2]. Kumar et al. (2014) tested various parameters to decrease bacterial populations in sludge, i.e., frequency (35 kHz and 130 kHz), power 250 watts and time period (5, 10, 20 and 30 minutes) [22]. As the frequency and time period increased, the bacterial population was decreased. It was also observed that a 130 KHz frequency was more effective than 35 kHz. Li et al. (2016) used ultrasonic waves with a frequency of 20 kHz with a power and irradiation time of 60 $W{\cdot}m^{-2}$ and 0 to 20 minutes, respectively. The rates of killing of E. coli is bigger than *S. aureus* [23].

The ultrasonic method has advantages over other methods to control MRSA, such as 1. It does not use antibiotics so that it does not cause antibiotic resistance; 2. It kills MRSA physically and chemically simultaneously and there is no chance to cause antibiotic resistance; 3. Uses relatively less power (maximum 8.4 watts) than similar methods in other research; 4. Physical proof of the death of MRSA and the causes can be clearly observed; 5. It has the potential to be developed as a wound debridement because it can selectively dissolve fibrin without harmful macroscopic changes in granulation tissue [24]. Many studies have used ultrasonic waves to kill bacteria, however, the mechanism of the death and study of the physical evidence of the dead bacteria have never been done. This research aims to find the lethal power of the ultrasonic wave exposure to kill MRSA in vitro while studying the mechanism of death using the indicators changes in shape, size, structure and Gram staining of MRSA cells.

MATERIALS AND METHODS

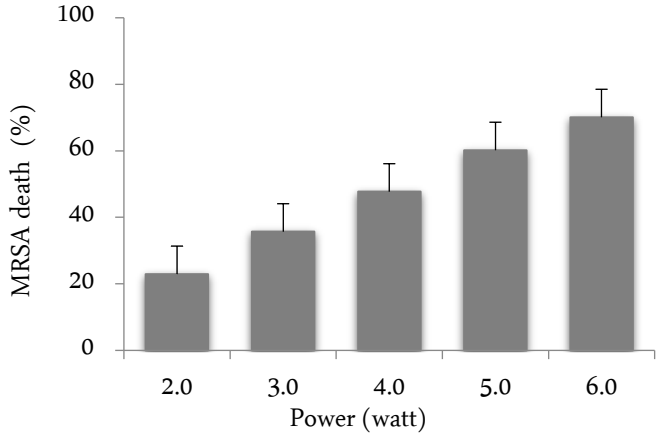
MRSA isolates were obtained from the Microbiology section of RS Dr. Soetomo, grown in Luria Bertani Broth Miller M1245-500G then diluted by graphic water. A total of 100 ml MRSA suspension exposed by an ultrasonic wave with frequency 26 [25] and a power of 2, 3, 4, 5, or 6 watts for 2 minutes. Then, the bacteria were cultured in a petri dish filled with Chromagar MRSA MR 500 POOO204, observing the grown MRSA population after 24 hours, using a Quebec colony counter, to create a regression equation to obtain a lethal power curve (100% cell death).

The macroscopic observation was done by the TPC method; microscopic observation using SEM was performed to understand changes in morphology of MRSA cells, changes in the structure of MRSA cells using TEM, and the changes in cell staining using Gram staining. This research was approved by the ethics committee of Medical Faculty of Wijaya Kusuma Surabaya University. No.485/SLE/FK/UWKS/IX/2014.

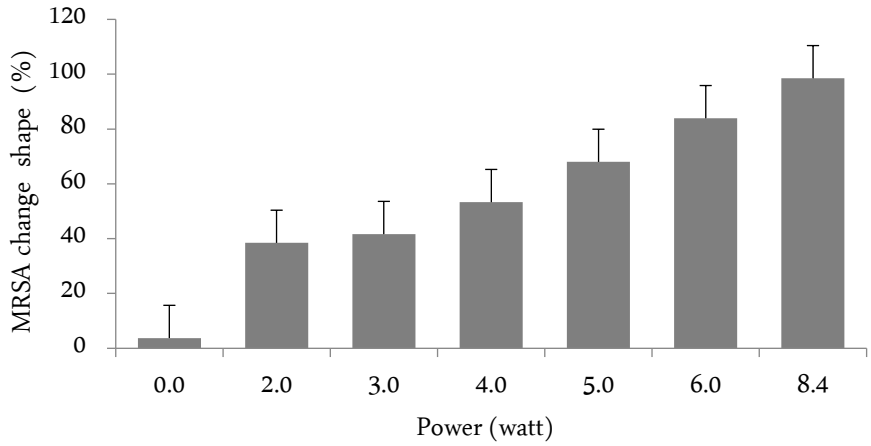
The death indicators of MRSA were changes in: shape, size, structure and cell staining, assessed via electron microscopy and Gram staining. The death percentage of MRSA (the amount of dead MRSA in a treatment divided by dead MRSA in the control multiplied by 100%) of the various treatments was statistically analyzed using ANOVA, while the more specific effects were tested via regression. Before performing the ANOVA test, the data were first tested for normality and homogeneity. The level of confidence used in all tests was $\alpha = 0.05$. The statistical analysis process used software program MINITAB 16.

RESULTS AND DISCUSSION

The result of the data analysis showed that the power of the ultrasonic waves had a significant effect on



(a)



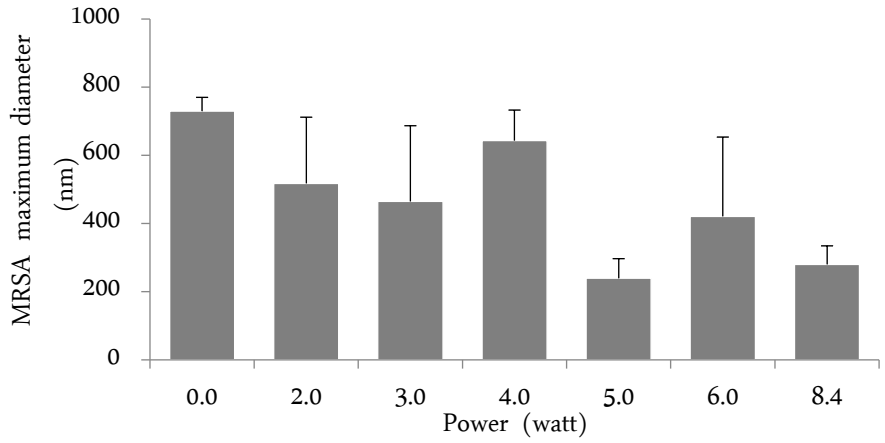
(b)

Figure 1. Data distribution of the effect of power ultrasonic wave exposure to the MRSA death (a). Data distribution of the effect of power ultrasonic wave exposure to the changes of MRSA shape. Shown that one of indicators of MRSA death is the change of MRSA shape (b).

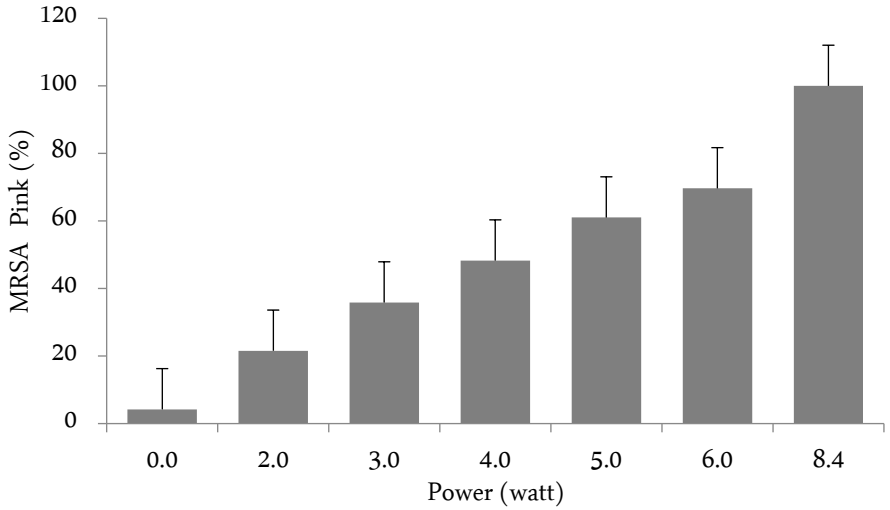
the death percentage of MRSA ($p = 0.000$), while the lethal power as found via regression was 8.432 watts. The death indicators of MRSA affected by ultrasonic wave are changes in shape ($p = 0.005$), size ($p = 0.70$); qualitatively there were changes in the structure of the cell and cellular uptake of Gram staining ($p = 0.000$), all compared with the control (Figure 1). The treatment of ultrasonic wave causing changes in the morphology and size of MRSA cell. Moreover, the ultrasonic wavelength also increases the MRSA death (Figure 2). The MRSA was stained with the Gram staining, the live cell color is purple but the death cell is a pink color.

This study we employed the square ultrasonic wave signal, which is induced more damage to the microbe compare to other signal forms [26]. This research is using direct contact method, which means the ultrasonic transducer is inserted into the bottom of the vessel which filled with MRSA suspension. The power of ultrasonic wave exposure (P) had a very significant effect on the death percentage of MRSA (K), with $p = 0.000$, and the effect as stated by regression equation: $K(P) = -0.188 + 11.881P$ and lethal power 8.432 watts. These results are supported by the indicators of death, as noted by MRSA cell observation with SEM and TEM. This lethal power is relatively small compared to the similar research using ultrasonic wave. The death of the cells happened due to necrosis.

The power of ultrasonic waves could disturb the lipid membrane, which affects bacteria growth or inhibits the growth process altogether [27], while the shift of mechanical power of the ultrasonic wave could cause the separation of multi-molecular complex and cause the



(a)



(b)

Figure 2. Data distribution of the effect of power ultrasonic wave exposure to the diameter of MRSA, statistically the power of ultrasonic does not affect the of MRSA ($p = 0.470$) but physically different (a). Data distribution of the effect of power ultrasonic wave exposure to the change color of MRSA that have died (pink MRSA) (b). From the data shown that the changes of MRSA are an indicator of MRSA death.

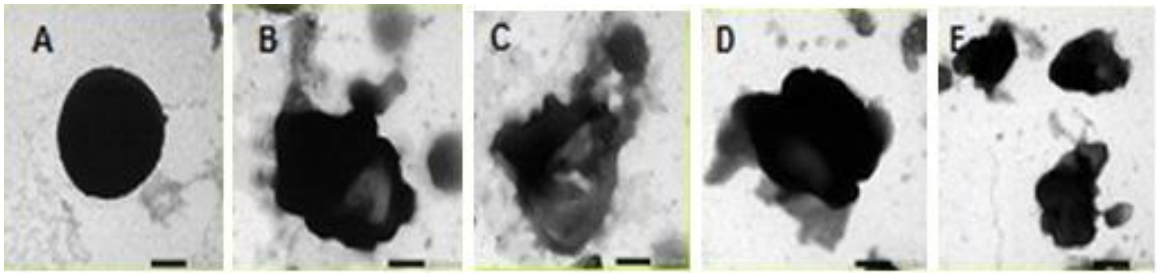


Figure 3. MRSA control (a, b, and c): Because of the chemical effect of H_2O_2 of MRSA cell wall which exposed by ultrasonic with 4 watt and 6 watts power is broken in the cell wall so the cytoplasm and organelles are out from the cell. Cell occurred mechanical stress was caused by ultrasonic exposed with 8.4 watts power, Pieces of cells occur because of the intracellular cavitation caused by ultrasonic exposure with 8.4 watts power (e). (Observed by TEM with magnification 20,000×, scale 200 nm)

stretch of the cell wall is beyond the elasticity limit, then the cell will be torn and the bacteria will die [20]. From the data analysis, it is shown that, when the MRSA was in the continuous ultrasonic field, periodic changes in pressure inflicts mechanical stress, which is marked by enlargement or shrinkage of the MRSA cell. If the elasticity limit of the MRSA cell wall is exceeded, the cell was torn, disrupting cell function and causing the MRSA cells to die (Figure 2).

Cavitation is regarded as the main mechanism that increases membrane permeability [28, 29, 30, 31]. Cavitation happens next to the surface; broken bubbles cause liquid with high velocity inflict shearing off next to the surface [32]. The extracellular cavitation causes a solid turbulation, while intracellular cavitation causes an explosion from the inside of the cell. When the suspension of MRSA was in a continuous ultrasonic wave field, then intracellular or extracellular cavitation occurred. When intracellular cavitation happens in the MRSA cells, then microbubbles will arise with increasing magnitude, which causes the density to decline so that the MRSA cells will keep moving to the surface of the suspension and make hydrostatic pressure decline. If the pressure inside the MRSA cell is higher than outside the cell, this will inflict an explosion that is marked by broken cell walls (Figure 3).

Free radicals are formed by continuous irradiation into MRSA suspension [20]. These free radicals with join with a hydrogen atom in the water so that will produce hydrogen peroxide (H_2O_2) [33]. Ultrasonic waves in liquid media caused mechanical effects (microstreaming, high shear force, shockwave) and sonochemical reactions (free radical, hydrogen peroxide), which finally caused interference or cell disruption of bacteria [34, 35, 36]. There are multiple effects of ultrasonic wave inhibition in microbe cells, including the formation of pores, thinning cell wall, interference with the cell membrane, release of cytoplasmic contents and damage to the DNA structure [37, 38]. If the suspension of MRSA exposed by ultrasonic wave continuously, hydrogen peroxide will react with MRSA cell walls so that the cell wall experiences thinning, which causes the cell to swell and in particular parts forming holes. Through these holes, the cytoplasm will leak out of the cell; if this happens continuously, then the cell will not be able to retain its form and will die. Combinations of various frequencies of ultrasonic waves with the various power of ultrasonic waves in a simultaneous experiment are needed to further characterize the optimum frequency and power to kill MRSA *in vitro*.

CONCLUSION

The power of wave influences very significantly to the death percentage of MRSA, with lethal power at 8.432 watts. Changes in shape, size, structure and cell staining due to mechanical stress, cavitation and chemical effect were observed in ultrasound-treated cells. Dead MRSA cell happened due to necrosis, will change in shape from round to not round, the mean of the maximum diameter can be smaller or larger from the mean of maximum diameter of control MRSA.

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