A Novel Method of Antibacterial Evaluation Based on the Inhibition of Hydrogen Sulfide Producing Activities of Salmonella –Using Copper as a Model Antibacterial Agent–*

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A novel method for detecting antimicrobial activity using an innate property of the *Salmonella* bacteria, namely, the ability of *Salmonella* to produce hydrogen sulfide (H_2S) was developed in this study. The validity of the method was evaluated by comparing the antibacterial activity of copper to that of aluminum. *Salmonella* was inoculated over the entire surface of deoxycholate hydrogen sulfide lactose (DHL) agar plates that included Ammonium ferric citrate (C_6H_8FeN). Approximately 25 μL of cupric chloride ($CuCl_2$, 1% weight ratio) solution or aluminum chloride ($AlCl_3$, 1% weight ratio) solution was added to the center of the medium. The surface of the medium was covered with polyethylene terephthalate (PET) films to induce an anaerobic state. *Salmonella* was cultured under anaerobic conditions at 310 K (37°C) for 86.4 ksec (24 h). The antibacterial activity of copper was determined by observing the medium surface color change due to iron sulfide (FeS) formation, which was caused by the production of H_2S by *Salmonella*; blackness indicated presence of newly formed FeS. A quantitative evaluation of copper's antimicrobial activity was performed using a gradient of $CuCl_2$ concentrations; results were compared with those of the present standard method, Kirby-Bauer disk diffusion method on the Mueller Hinton medium. Finally, in order to evaluate the antibacterial activity of metals, *Salmonella* was inoculated on DHL agar plates. Subsequently, Japanese coins (1-yen, 5-yen, 10-yen, 50-yen, 100-yen, and 500-yen coins) were placed on the agar and cultured at 310 K for 86 ksec. *Salmonella* cultured in the presence of $AlCl_3$ produces black color, while no blackening is observed with $CuCl_2$, suggesting that copper possesses an antibacterial property against *Salmonella*. $CuCl_2$ suppresses H_2S production by *Salmonella*, as copper ions form a transparent circle or ellipse (new halo) around the point at which $CuCl_2$ had been plated. The size of the new halo increases in direct proportion to the concentration of $CuCl_2$. The halo is no longer visible at 0.034 mg of $CuCl_2$ in our method, while the halo disappears with 4.34 mg of $CuCl_2$ in the Kirby-Bauer test. Therefore, the present method is 129 times more sensitive than the standard method, suggesting increased usefulness and effectiveness in testing antibacterial activity. No FeS-dependent black circle is formed under any of the coins, with the exception of the 1-yen coin, which contains aluminum and no copper. Therefore, the copper-containing coins have an antibacterial effect. [doi:10.2320/matertrans.M2016007]

(Received January 15, 2016; Accepted March 10, 2016; Published April 8, 2016)

Keywords: salmonella, copper, antimicrobial, hydrogen sulfide, iron sulfid

1. Introduction

Among metals with antibacterial activities, silver and copper have long been known for their toxicity towards microorganisms but relative safety to the human body. On the other hand, titanium, which is recognized as a promising medical material for implants and other purposes, and most familiar metals including iron and aluminum, do not have antibacterial properties in themselves^{1–3}. The antibacterial activities of metals are not generally expressed by themselves but by the metallic ions derived from them.

Today, a wide range of antibacterial products is available in the market, including kitchen accessories to enhance food hygiene, clothing such as underwear and stockings, and bath/toiletry goods. The method to evaluate the antibacterial activity and efficacy on the surface of these plastic/metal/ceramic products with antibacterial finishing is defined by the Japan Industrial Standards (JIS) Z 2801: 2010⁴. The specified method for evaluation of the antibacterial activities of a material involves the following steps: bringing the subject in contact with a solution containing the gram negative *Escherichia coli* (*E. coli*) or the gram positive *Staphylococcus aureus*, culturing the mixture on agar medium available in market at 310 K (37 degrees C) for 86.4 ks (24 h), counting the number

of viable bacteria contained in 1 mL of the solution, and comparing the result with the bacterial count of the control group that was not allowed the bacterial solution to contact with the subject. However, this method is cost- and labor-intensive due to the requirements of solution preparation and colony counting. Therefore, the authors designed an alternative method exploiting the hydrogen sulfide (H_2S)-producing property of *Salmonella* bacteria.

The *Salmonella* bacterium, one of the causative agents of food poisoning, is characterized by its significant production of H_2S during growth. H_2S produced by *Salmonella* reacts with iron (Fe), if present in the culture medium, to form black colored iron sulfide (FeS). Therefore, agar medium containing Fe in the form of iron citrate shows color change upon H_2S production by *Salmonella*, thus allowing visual evaluation of antibacterial activity. FeS is formed in mildly acidic conditions and is dissolved in strong acid/alkali⁵. These characteristics of *Salmonella* allow antibacterial evaluation simply by verifying the formation of black FeS, and are therefore expected to decrease the cost and labor involved in determining antibacterial activities. The simple procedure of culturing H_2S -producing bacteria including *Salmonella* will eliminate the necessity of counting viable bacteria for statistical comparison with the control group.

Therefore, this study evaluates the antibacterial activities of copper ions with this novel antibacterial evaluation method to investigate its potential. The current method used for test-

*This Paper was Originally Published in Japanese in J. Japan Inst. Met. Mater. **80** (2016) 165–170. The title of the paper is changed.

ing antibacterial sensitivity, or the Kirby-Bauer test⁶⁾ was used as the control method instead of the method specified by JIS (JIS Z 2801 : 2010⁴⁾) because the latter excludes the use of strains other than *E. coli* and *Staphylococcus aureus*. However, the Kirby-Bauer test imposes no restraint on the strains to be used, allowing the use of *Salmonella* for direct comparison of test results with the same subject microorganism.

2. Experimental Procedures

2.1 Bacterial strain and culture medium

Non-typhi *Salmonella enterica subsp. enterica serovar Virchow*⁷⁾ that produces a large amount of H₂S was used as a bacteria strain in this study.

Deoxycholate-hydrogen sulfide-lactose (DHL) medium (Eiken Chemical, Japan) was used as the culture medium. This medium does not require any sterilization and is characterized by the presence of iron citrate (C₆H₅FeO₇) as the iron source, and sodium thiosulfate (Na₂O₃S₂) as the source of sulfur. In this medium, the metabolism of *Salmonella* results in the release of sulfur from sodium thiosulfate and production of H₂S, which reacts with Fe contained in iron citrate to form the black-colored FeS. The resulting color change to black indicates the detection of the strain. For preparation of DHL media, 63 g of powdered medium was dissolved in 1,000 mL of distilled water, maintained at 373 K, and subsequently 20 mL of the solution was dispensed in an 80-mm petri dish, and allowed to solidify at room temperature. Multiple dishes were prepared. For the control Kirby-Bauer test⁸⁾, 38 g of powdered Mueller-Hinton medium (Eiken Chemical) was dissolved in 500 mL of distilled water. The solution was sterilized at high pressure and high temperature for 1.2 ksec at 394 K, and 20 mL of the solution was dispensed in a petri dish, and solidified at room temperature. Multiple dishes were prepared.

2.2 Subject metals to evaluate antibacterial activity

Solution of copper and aluminum ions for qualitative and quantitative evaluation of antibacterial activity were used in this study. The detailed procedures are as follows.

Qualitative testing: 1 g of copper (II) chloride dihydrate (CuCl₂ · 2H₂O), which is a copper ion compound with antibacterial activities, was dissolved in 100 mL of distilled water to prepare a 1% solution (0.06 mol). As a control, 1 g of aluminum chloride (AlCl₃ · 6H₂O), which has no antibacterial property, was dissolved in 100 mL of distilled water (0.04 mol).

Quantitative testing: 7 g of CuCl₂ · 2H₂O was dissolved in 10 mL of distilled water (4.1 mol:to form a nearly saturated solution), and was then subjected to two-fold serial dilution to prepare solutions of 0.6836, 1.367, 2.734, 5.469, 10.94, 21.88, 43.75, 87.5, 175, 350, and 700 mg/mL.

2.3 Culture method under anaerobic conditions

Anaerobic conditions were induced using a transparent polyethylene terephthalate (PET) film that was sterilized at high temperature 394 K under high pressure for 1.2 ks. The 0.2-mm thick PET film was cut into squares of 20 mm and 50 mm, which were placed in close contact with the medium surface to block the air and establish an anaerobic state.

2.4 Procedure for the quantitative and qualitative evaluation of antibacterial activities

The procedure comprises the following four steps.

(1) Preparation of *Salmonella* culture: The strain was inoculated on a DHL agar plate with a nichrome loop, and incubated at 310 K for 86.4 ks to obtain a discrete colony of *Salmonella*. The colony was picked with a cotton swab tip and applied to the entire surface of the DHL agar medium in a fresh petri dish.

(2) Qualitative evaluation: A 0.2-mm thick 20-mm square transparent PET film was immersed in 1% CuCl₂ solution and 1% AlCl₃ solution, respectively to evaluate the difference in antibacterial activities between copper ions and aluminum ions. The film was dried and placed in close contact with the DHL agar medium to generate an anaerobic condition and the culture was incubated at 310 K for 86.4 ks. As a control, a 20-mm square transparent film with no treatment was incubated in the same manner as the test culture.

(3) Quantitative evaluation: To the center of the DHL agar medium, 25 μL of CuCl₂ solutions of concentrations ranging from 0.6836 mg/mL to 700.0 mg/mL were dropped. The spot was then covered with a 0.2 mm-thick 50-mm square PET film to generate an anaerobic condition and was then incubated at 310 K for 86.4-ks.

The control Kirby-Bauer test was performed as follows:

As indicated above, 25 μL each of CuCl₂ solutions of varied concentrations were dropped on a 10-mm paper disk (Blaine test paper, ADVANTEC, Japan). The disk, infiltrated with CuCl₂ solution, was then placed on the Mueller-Hinton agar plate entirely inoculated with *Salmonella* and was incubated at 310 K for 86.4 ks. The PET film was not used because the Kirby-Bauer test does not require anaerobic conditions on the medium surface.

(4) Evaluation of antibacterial activities

The DHL agar media inoculated with *Salmonella* showed blackening due to FeS formation in the anaerobic region, but no change in the region where the antibacterial metal ion solution was added because H₂S was not produced in that area. The area that showed no blackening was circular or elliptical shape because the metal ion solution had spread radially. The surface area of this circular or elliptical shape was measured as an index of the strength of the antibacterial effect. On the other hand, in the control Kirby-Bauer test using the Mueller-Hinton agar, a round zone of inhibition was formed around the disk after incubation where no bacterial growth was observed. The diameter of this inhibition was also measured (according to the current standard method).

2.5 Application to an antibacterial test of metals

The Japanese 1-yen coin of aluminum, 5-yen coin of copper-zinc alloy, 10-yen coin of copper-tin alloy, and 50/100/500-yen coins of nickel-copper alloy were placed on the DHL agar media entirely inoculated with *Salmonella*. A transparent 0.2-mm thick 20-mm square PET film was used as the control. The coins were incubated on the medium at 310 K for 86.4 ks, and were then removed to compare the change in the medium color underneath.

3. Results and Discussion

3.1 Effects of anaerobic conditions on the formation of FeS by *Salmonella*

Figure 1 shows a plate containing the DHL agar medium entirely inoculated with *Salmonella* and incubated under a PET film at 310 K for 86.4 ks. Growth of *Salmonella* is detected by blackening of the medium under anaerobic conditions generated by the PET film. A typical characteristic of non-typhi *Salmonella* is the abundant production of H_2S using sulfur sources such as thiosulfuric acid present in the DHL medium. Active growth of *Salmonella* is therefore confirmed by blackening of the medium due to FeS formed by the reaction between H_2S produced by *Salmonella* and iron (III) citrate included in the medium. Since the PET film itself is not antibacterial in nature, active growth of *Salmonella* is observed underneath it. However, the area that is not covered with the PET films does not exhibit any blackening. Probably because the FeS generated is oxidized again in the aerobic condition. Exposure to oxygen supply in the air resulted in loss of black color. On the other hand, FeS generated from H_2S does not seem to be re-oxidized in the anaerobic condition where oxygen supply from the air is blocked by the PET film.

3.2 Antibacterial activities of copper ions and aluminum ions

Figure 2 shows a plate containing the DHL agar medium entirely inoculated with *Salmonella* and incubated under a PET film at 310 K for 84.6 ks. The PET film impregnated with copper ions or aluminum ions was placed on the medium surface to induce an anaerobic condition. Blackening is confirmed under the PET film treated with aluminum ions whereas no blackening is observed under that treated with copper ions. These color reactions indicate that FeS is generated in the presence of aluminum ions that do not inhibit H_2S pro-

duction by *Salmonella* whereas it is not generated in the presence of copper ions due to inhibition of H_2S production. This indicates that copper ions have antibacterial effects whereas aluminum ions do not, which is a known fact¹⁻³⁾. Therefore, this study demonstrated for the first time that antibacterial activity can be determined by the formation of FeS due to the H_2S produced by *Salmonella*.

3.3 Comparison of antibacterial activity of copper ions as quantified by the novel method and the current standard method

Figure 3 shows the results of antibacterial activity testing by our proposed method (upper row) and by the current standard Kirby-Bauer method (lower row). In the Kirby-Bauer method, the inhibition zone representing zero bacterial growth appears at a copper ion concentration ≥ 4.37 mg. On the other hand, in the novel method, the inhibition zone is shown as a circle or an ellipse appears at a copper ion concentration ≥ 0.0034 mg. We have designated the region under the PET film without the black FeS formation as “new halo” to indicate inhibition by the novel method, and the zone of inhibition obtained by the standard method as “old halo.” The size of the new halo was found to form in proportion to copper ion concentration. The surface under the PET film outside the new halo exhibits blackening and indicates FeS formation whereas the surface outside the PET film does not show any change in color.

The threshold for the new halo formation was 0.034 mg, which is far lower than 4.37 mg that is obtained with the conventional method, and is equivalent to a ratio of approximately 1/129. Thus, the novel method quantitatively displayed higher sensitivity for evaluation of antibacterial activity than the current method did. In addition, when a halo is formed in the standard method, the microorganisms under the paper disk are dead and their growth cannot be visually determined. In contrast, the new halo has a distinct black-white contrast that allows clearer visual evaluation of bacterial growth. Fig-

Fig. 1 Iron sulfide formation by the reaction between hydrogen sulfide produced by *Salmonella* and iron (III) citrate in the medium under anaerobic conditions.

Fig. 2 Effects of copper ions and aluminum ions on FeS formation by *Salmonella*.

Fig. 3 Comparison of sensitivity between the new method and the Kirby-Bauer method under CuCl_2 treatment. Upper: New method using the DHL medium. Lower: Kirby-Bauer method using the Mueller Hinton medium.

Fig. 4 Comparison of the formed zone of inhibition between the new method and the Kirby-Bauer method. ■: New method. ■: Kirby-Bauer method.

ure 4 shows a graph comparing the surface area in mm^2 between the new and old halos with the varied copper ion concentrations shown in Fig. 3. The size of the halo size constitutes another difference between the new and current standard method, with the new halo having a surface area that is 3 to 10 times larger than that of an old halo.

3.4 Superiority of the method proposed in this study

Our previous study demonstrated that H_2S production is hampered when saline concentration in the medium is increased despite continued growth of *Salmonella*⁹⁾. Similarly,

the results of this study simply imply that the copper ions may decrease the H_2S productivity of *Salmonella* but that they may not necessarily stop the bacterial growth. Therefore, a decrease in FeS formation related to H_2S production may reflect a lowered rate of multiplication in *Salmonella*.

The current standard method uses ordinary agar or Mueller-Hinton agar that contains no sulfur or iron source that enables H_2S production or FeS formation by the microorganisms. Therefore, determination of growth inhibition in microorganisms requires visual evaluation of the old halo. The minimum concentration of antibacterial materials at

Fig. 5 Effects of coins on *Salmonella* growth. (¥1: 1-yen coin, ¥5: 5-yen coin, ¥10: 10-yen coin, ¥50: 50-yen coin, ¥100: 100-yen coin, ¥500: 500-yen coin, PET: Plastic film)

which the formation of the old halo can be visually recognized is called the minimum inhibitory concentration (MIC) which, in this study, was proved significantly higher than the minimum concentration required blocking H_2S production in the novel method.

These results imply that the novel method has a higher sensitivity than the current standard method and enables distinct determination of microorganism growth inhibition effects.

3.5 Application to antibacterial activity testing using coins

Figure 5 shows the results of antibacterial activity evaluation by the new method. The coins were kept in close contact with the agar such that no air could intrude between the coins and the agar. The blackening underneath the coins due to FeS formation was demonstrated only for the 1-yen coin and the control PET film sheet. Color changes for other coins that were similar to blackening were due to rust. These results indicate the antibacterial effects of copper, which is present in all the coins other than the 1-yen coin. The 1-yen coin is made of aluminum and, therefore, did not exhibit the antibacterial effect seen in the other coins. This simple method of application clearly demonstrates antibacterial activity of copper and is expected to lower the cost and labor required for antibacterial testing. The existing DHL agar was used in this study, but in order to improve the sensitivity further, media with better sensitivity can be developed specifically for the visual evaluation of FeS formed by *Salmonella*. Further, the copper ion used in this study resists quality degradation (titer decrease) better than organic antibacterial agents including penicillin and other antibiotics. The toxicity of copper in humans that was discussed in the past has now been denied¹⁰⁾, thus proving the suitability of copper for a wide range of applications from the perspective of safety. This new evaluation method can be applied to the testing of a wide range of antibacterial metals and materials other than copper. The higher sensitivity of this novel method may serve the discovery of antibacterial substances that may have been overlooked by use of the conventional method.

This novel method can be used to test a wide range of products and reagents for antibacterial properties, including dishes, cleaners, and antibiotics. We will continue to investigate these possibilities in the future.

4. Conclusions

A new method of antibacterial activity evaluation by exploiting formation of FeS due to H_2S produced by *Salmonella* was developed and proved the effectiveness of the novel method using copper, which has known antibacterial properties in this study. In addition, the results of the novel method were compared with those of the current standard method Kirby-Bauer test. The following results were obtained.

(1) *Salmonella* was inoculated entirely over the DHL agar medium containing sources of sulfur and iron. A PET film sheet was placed over part of the medium to induce an anaerobic condition and the dish was incubated at 310 K for 86.4 ks. The anaerobic part of the medium was blackened with the FeS formed due to the reaction between H_2S produced by *Salmonella* and Fe contained in the DHL agar.

(2) Using a copper as an antibacterial substance and aluminum as the control substance, the part of medium covered with the PET film containing aluminum ions was blackened, whereas that with the PET film containing copper ions shows no blackening. Thus, the antibacterial property of copper was visually determined.

(3) In the test that used copper ion solutions of differing concentrations, the zone of inhibition was formed with its center at the point where the copper solution was dropped and the area of the circle was proportional to the copper ion concentration. A comparison of the novel method with the standard Kirby-Bauer test suggested that the former method had a superior sensitivity equivalent to 129 times that of the current standard method. The surface area of inhibition is also 3 to 10 times larger with the novel method than that of the method.

(4) Different coins that are in circulation in Japan were placed each other on the DHL agar medium entirely inoculated with *Salmonella*. The blackening of medium due to FeS formation was observed only under the 1-yen coin made of aluminum. All other coins contain copper, and therefore showed antibacterial activities.

(5) This novel and simple method for highly sensitive evaluation of antibacterial activity was developed based on FeS formation using *Salmonella* and was proposed for the first time in this study. Further improvement of the method or development of other methods that apply this principle can be expected to improve the productivity and performance of the antibacterial evaluation.

Acknowledgment

This study was a joint research effort with the Institute for Materials Research, Tohoku University (Grant number 14K0005, 15K0018) and was funded by JSPS Kaken with Grant number 15K00894 to which we sincerely express our gratitude.

The manuscript was edited and proofread by a translation agency, a priori, Inc. Tokyo, Japan.

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