A simple method for evaluating the antimicrobial capacity of insoluble powdered inorganic materials

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Abstract

Although several methods have been devised for determining the antimicrobial ability of various materials, they suffer from various limitations. For example, it is difficult to determine the antimicrobial ability of poorly water-soluble materials. This study proposes a simple method for evaluating the antimicrobial ability of insoluble powdered metal oxides based on the minimum inhibition concentrations (MICs) determined by combining the halo method, a common method for evaluating the antimicrobial ability, with the flat-plate dilution method. Using Al_2O_3 powder, which did not exhibit antimicrobial properties, as a diluent, we prepared a uniform mixture of Ag_2O, CuO, and ZnO at several concentrations (ppm). We then tested the antimicrobial activity of these powders for antimicrobial activity against *E. coli* using the halo method. Our method can be highly useful in evaluating the antimicrobial ability of poorly water-soluble inorganic powdered materials.

Key words

agar zone of inhibition method, MIC value, flat-plate dilution method, film-adhesion method, *E. coli*

1. Introduction

Silver, copper, and zinc exhibit excellent heat resistance and can be incorporated into plastics. Hence, they are extensively utilized to prepare inorganic antimicrobial agents. Their antimicrobial mechanism involves the elution of metal ions on bacterial cell membranes and membrane proteins, inhibiting bacterial growth. The film-adhesion method (JIS Z2801: 2010) is a quantitative method for evaluating the antimicrobial susceptibility of antimicrobial samples. In this method, a bacterial suspension is dropped onto the surface of an antimicrobial sample and covered with a thin, sterile film. After 24 h, the bacteria are collected from the sample and the viable bacterial count is measured. The number of viable bacteria is compared to that of the control (untreated) to quantify the antimicrobial effect. A wide range of materials can be used as antimicrobial samples, including ceramics, metals, plastics, paints, rubber, wood, and paper (Sjollema et al., 2018; Matsumoto et al., 2020). However, it is difficult to prepare an antimicrobial sample with poorly water-soluble antimicrobial agents.

The antimicrobial activity of a liquid is determined based on the minimum inhibitory concentration (MIC) (Taylor et al., 2020; Ross et al., 2013). The MIC is the minimum concentration of an antimicrobial agent that will inhibit the growth of a microorganism in a culture medium after overnight incubation. The MIC is typically determined using the flat-plate dilution method. In this method, the antimicrobial agent to be tested is first mixed with an agar medium and placed in a Petri dish to prepare agar plates. Several concentrations of antimicrobial agents are prepared. The agar medium is then coated with a solution of *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* and left to incubate overnight. The concentrations that did not result in a visual growth of microorganisms are known as susceptibility values (MIC values). The reported MIC values for *E. coli* are 1.0 ppm for Ag^+, 15.7 ppm for Zn^{2+}, and 39 ppm for Cu^{2+} (Kikuchi, 2000). However, it is difficult to use the flat-plate dilution method to determine the MIC of water-insoluble solid powders, because the antimicrobial agent will settle out owing to the difference in the specific gravity with the agar medium until solidified. Consequently, only a small amount of the antimicrobial agent gets exposed on the surface of the agar medium, making it impossible to determine the accurate MIC value.

In contrast to the MIC method, the halo method, also known as the agar zone of inhibition method (JIS L1902), is a qualitative method for evaluating the antimicrobial susceptibility of water-insoluble substances. In this method, microorganisms are inoculated in a molten agar medium, which is then allowed to solidify. Next, the samples are placed on the inoculated and solidified agar medium and left to incubate for 24-48 h. If the sample contains an antimicrobial component that inhibits bacterial growth, an area (inhibition zone or "halo") is formed around the sample in which the bacteria do not grow. The antimicrobial effects are qualitatively evaluated based on the presence or absence of such halos (Naresh et

al., 2020; Mohanty et al., 2023).

Although the film-adhesion and flat-plate dilution methods based on MIC values are excellent for evaluating the activity of antimicrobial agents, they are not suitable to achieve a uniform dispersion of the antimicrobial agent in the sample when applied to solid powdered antimicrobial agents that are not water-soluble. This study proposes a method to determine the MIC value by combining the halo method with the flat-plate dilution method to evaluate the antimicrobial ability of inorganic powdered materials.

2. Experimental procedure

2.1 Preparation of culture media and antimicrobial susceptibility testing

Bacto tryptone was obtained from Difco. The yeast extract was acquired from Nacalai Tesque, Inc. Both agar and NaCl (a special-grade reagent) were from Fujifilm Wako Pure Chemicals Co. Sterile polystyrene Petri dishes (diameter: 90 mm, height: 15 mm) were obtained from Azwan Co. Personal-11 thermostatic shaking chamber was acquired from Titech Co. CR-32 thermostatic incubator was from Hitachi Ltd.

2.2 LB agar medium

To prepare an LB agar medium, 5.0 g of bacto tryptone, 2.5 g of yeast extract, 10 g of agar, and 2.5 g of NaCl were dissolved in 500 mL of distilled water and sterilized at 121 °C for 15 min at 2 atm (Table 1) (Luria et al., 1957). This solution was then cooled slightly at room temperature and transferred to a Petri dish and solidified.

2.3 *E. coli* culture

To prepare an *E. coli* culture, 0.2 g bacto tryptone, 0.1 g NaCl, and 0.1 g dried yeast extract were dissolved in 20 mL of distilled water and transferred to a 100-mL volumetric triangular flask. After sterilization at 2 atm for 15 min at 121 °C, the liquid medium was allowed to cool to room temperature. Then, *E. coli* (NBRC 3301) was inoculated with a sterilized platinum loop and incubated at 30 °C for 24 h at 110 rpm with shaking.

2.4 Inoculation of *E. coli* on the LB agar medium

First, 100 μL of 10^5-fold dilution of the liquid medium was collected. It was then inoculated onto the LB agar medium and spread by a conlage rod.

2.5 Test-molded body and antibacterial tests

A green compact obtained by press-molding the antibacterial test powder at 100 MPa using a stainless-steel mold with a diameter of 10 mm was placed on the top of an LB agar medium inoculated with *E. coli*. The growth status of *E. coli* was observed after being left in a constant-temperature bath (30 °C) for 24 h.

2.6 Metal oxides and hydroxides

Eleven divalent-to-hexavalent metal oxides and six hydroxides (Table 1) were selected as solid, water-insoluble powders to be tested for their antibacterial activity against *E. coli*. Al_2O_3 (AKP-3000 grade) was obtained from Sumitomo Chemical Co., Ltd. ZrO_2 (EP grade) and $Zr(OH)_4$ were acquired from Daiichi Kigenso Kagaku Kogyo Co., Ltd. Other special-grade reagents were obtained from Fujifilm Wako Pure Chemicals Co. The research method is summarized in Figure 1.

3. Results and discussion

Among the aforementioned divalent-to-hexavalent metal oxides and hydroxides, AgOH, $Cu(OH)_2$, $Zn(OH)_2$, $Nb(OH)_5$, and $Mo(OH)_6$ were not tested for antimicrobial activity because they are unstable in the atmosphere at room temperature. A halo indicates the presence of antimicrobial activity, the strength of which can be indicated by the diameter of the zone of inhibition (halo diameter). Those that did not exhibit any halo, indicating the absence of antimicrobial activity, were labeled "None." Compacts of 1-cm diameter were used in the tests. In addition to Ag_2O, CuO, and ZnO, antimicrobial properties were observed for CaO and MoO_3 too. In the CaO compact, a bulge (diameter: 1.2 cm) was observed during the test, which is believed to form when a part of CaO converted to $CaCO_3$ and/or CaOH. This phenomenon also explains why the influence of the halo diameter of CaO is greater than that of the others. Ag_2O, CuO, ZnO, and CaO generate reactive oxygen species. Hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and superoxide ($:O_2$) are generated when ionic species from each powder are dissolved in minute quantities. These species are believed to be involved in the antimicrobial activity (Sawai et al., 1996). In addition, it has been reported that MoO_3 is influenced by the LB agar medium during testing, hydroxyl radical ions (H_3O^+) are generated through a two-step chemical reaction ((1) $MoO_3 + H_2O \rightleftharpoons H_2MoO_4$, (2) $H_2MO_4 + 2H_2O \rightleftharpoons 2H_3O^+ + MoO_4^{2-}$), and that the generated H_3O^+ de-

Table 1: Antibacterial activity and diameter of zone of inhibition (cm) of *E. coli* upon using raw reagents

Ag^{I}_2O	3.0 cm	–	–
$Mg^{II}O$	None	$Mg^{II}(OH)_2$	None
$Ca^{II}O$	3.2 cm	$Ca^{II}(OH)_2$	None
$Ni^{II}O$	None	$Ni^{II}(OH)_2$	None
$Cu^{II}O$	2.0 cm	–	–
$Zn^{II}O$	2.7 cm	–	–
$Al^{III}_2O_3$	None	$Al^{III}(OH)_3$	None
$Fe^{III}_2O_3$	None	$Fe^{III}O(OH)$	None
$Zr^{IV}O_2$	None	$Zr^{IV}(OH)_4$	None
$Nb^{V}_2O_5$	None	–	–
$Mo^{VI}O_3$	2.1 cm	–	–

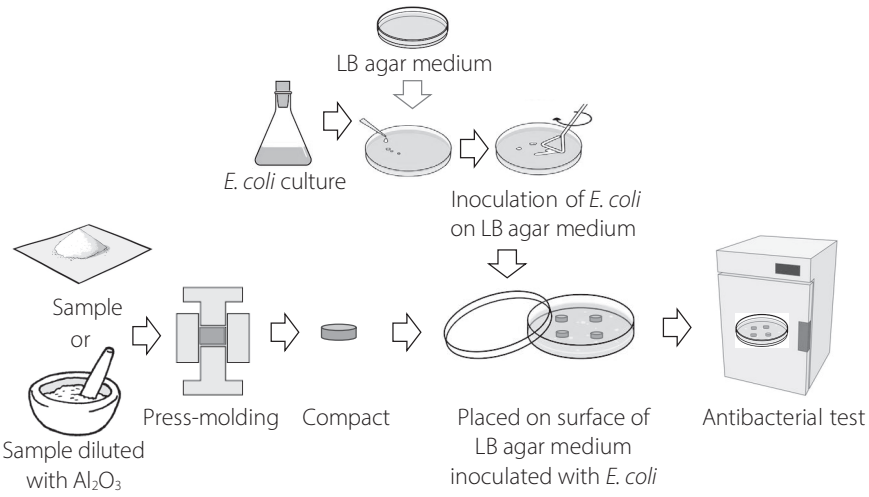

Figure 1: Procedure for evaluating the antimicrobial capacity of insoluble powdered inorganic materials

creases the pH (Matsumoto et al., 2019).

Next, we determined the MIC values of Ag_2O, CuO, and ZnO, which possess antimicrobial properties. Al_2O_3 (AKP-3000) powder, which does not exhibit antimicrobial properties, was used as the diluent. Ag_2O, CuO, and ZnO were each mixed with Al_2O_3 to achieve the concentrations (ppm) shown in Table 2. They were then thoroughly mixed for 1 h in a mortar. The mixed powder was then compacted by pressure molding to 100 MPa in a 10-mm-diameter stainless-steel mold and tested for antimicrobial activity. Figure 2 shows the photographs of (a) 100-ppm Ag_2O, (b) 1,000-ppm Ag_2O, (c) raw reagent Ag_2O compact, and (d) raw reagent Ag_2O powder after antimicrobial testing. The compacts ((a) 100-ppm Ag_2O, (b) 1,000-ppm Ag_2O, and (c) raw reagent Ag_2O), which are pressed powders, exhibited a halo in which the growth of *E. coli* was prevented. However, it is difficult to observe a similar halo in (d) (raw reagent Ag_2O powder), which is not pressed. Hence, it is essential to prepare compacts of samples for antimicrobial susceptibility testing. The MIC values of Ag_2O are much smaller than those of CuO and ZnO because of the difference in solubility in water; Ag_2O is an insoluble solid powder; however, its solubility in 100 g of water is 25 mg (Lide, 2004: 4-38). Although it exhibits trace solubility in water, its solubility is still considered one to two orders of magnitude higher than those of CuO and ZnO. The high amount of metal ions involved in the antimicrobial properties that dissolve in the LB agar medium could be a determining factor for the antimicrobial capacity. The MIC values for Ag_2O, CuO, and ZnO were 1-4 orders of magnitude higher than those of liquid antimicrobial agents (Ag^+: 1.0 ppm, Zn^{2+}: 15.7 ppm, Cu^{2+}: 39 ppm) (Kikuchi, 2000), probably because of the difference in the number of metal ions involved in the antibacterial property.

We believe that our evaluation method may allow effective and rapid screening of new antibacterial materials. In addi-

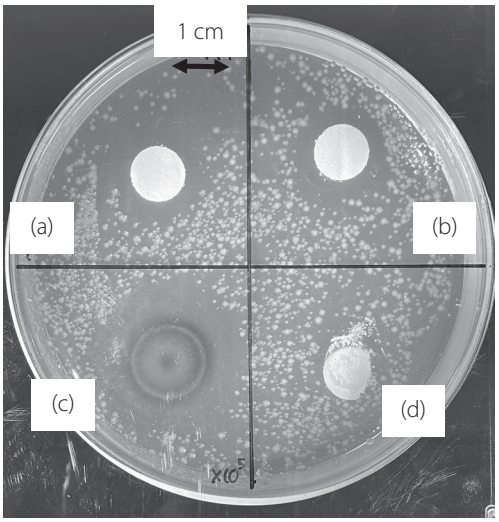

Figure 2: Zone of inhibition of (a) 100 ppm of Ag_2O, (b) 1,000 ppm of Ag_2O, (c) raw reagent Ag_2O compact, and (d) raw reagent Ag_2O powder

Table 2: Antibacterial activity and diameter of zone of inhibition (cm) of *E. coli* upon using 10-100 ppm of Ag_2O, 1,000-100,000 ppm of CuO, and 100-100,000 ppm of ZnO diluted with Al_2O_3

	Raw reagent	Concentration of raw reagent for Al_2O_3 (ppm)					
		100,000	10,000	1,000	100	50	10
Ag^{I}_2O	3.0 cm	–	–	–	2.2 cm	1.9 cm	None
$Cu^{II}O$	2.0 cm	1.1 cm	None	None	–	–	–
$Zn^{II}O$	2.7 cm	2.0 cm	1.7 cm	None	None	–	–

tion, it can be applied to both Gram-negative bacteria (such as *E. coli*, *Pseudomonas aeruginosa*, *Salmonella*, and *Bacillus cereus*) and Gram-positive bacteria (such as *Staphylococcus aureus* and *Proteus mirabilis*) (Rajivgandhi et al., 2018; Naresh et al., 2020; Rajagopalachar et al., 2022). However, it is difficult to apply this evaluation method to anaerobic bacteria. We also believe that it can be applied to mold/filamentous fungi, which are the same microorganisms as bacteria. We considered the lower limit concentration of the MIC to be approximately 10 ppm. We prepared the samples in 5-10 g to determine their MIC values. However, 100 g (Al_2O_3: 99.9990 g, Ag_2O: 0.0010 g) of the sample was prepared when the MIC was 10 ppm. We believe that a weighing value of 0.0010 g is the limit of accuracy that can be supported by a general chemical balance with a minimum readability of 0.0001 g.

4. Conclusion

The currently available inorganic antimicrobial agents mainly consist of metallic components such as silver, copper, and zinc. They exhibit excellent heat resistance, can be incorporated into plastics, and are used in various applications. However, it is difficult to evaluate their antimicrobial ability because of the difficulty in uniformly dispersing insoluble powdered substances, such as metal oxides, in the test material. We selected 11 oxides and 6 hydroxides as insoluble powdered materials. The antibacterial activity of insoluble powdered substances was simply evaluated based on the minimum inhibitory concentration (MIC) determined by combining the halo method, a common method for evaluating the antimicrobial ability, with the flat-plate dilution method. We first prepared the compacts of the aforementioned 17 materials and then examined their antimicrobial activity using *E. coli* by the halo method. Using Al_2O_3 powder, which did not exhibit antimicrobial properties, as a diluent, we prepared a uniform mixture of Ag_2O, CuO, and ZnO, which exhibited antimicrobial properties, at several concentrations (ppm). The minimum concentration at which these compacts demonstrated antibacterial activity was taken as the MIC value. We believe that our method can be used for evaluating the antimicrobial ability of powdered inorganic substances. This method can also be applied in various other fields.

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