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Susceptibility of *Mutans streptococci* in the Planktonic and Biofilm State to Erythrosine

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Abstract

The aim of this study was to investigate the susceptibility of *Mutans streptococci* in both planktonic and biofilm states to erythrosine.

S. mutans was cultured in brain-heart infusion (BHI) broth. Erythrosine was diluted in BHI broth and prepared at a concentration range of 0.02 - 10000 μg/L. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured using the microdilution method. After forming biofilms on 96-well plates, the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were measured.

S. mutans was susceptible to erythrosine in both planktonic and biofilm states. MIC and MBC values were both 19.5 μg/L for the planktonic state, while MBIC and MBEC values were 313 μg/L and 2500 μg/L, respectively, for the biofilm state.

Erythrosine (19.5 μg/L) exhibited a bactericidal effect on *S. mutans* (killing 99.9%) in the planktonic state. For biofilms, erythrosine inhibited biofilm growth and eradicated 99.9% of biofilm bacteria at higher concentrations than MIC and MBC. These MBIC and MBEC concentrations are much lower than known noxious doses, and the MIC, MBC, and MBIC values were even lower than clinical concentrations.

Key words : Erythrosine, *Mutans streptococci*, Biofilm, Susceptibility

Ⅰ. Introduction

Dyes have been used for oral hygiene instruction in dental offices[1]. Erythrosine, also known as FD&C Red #3, is one of dye for dental office. It is the disodium salt of 2,4,5,7-tetraiodofluorescein and expresses the color of cherry-pink[2]. 0.72 - 2% (w/v) aqueous solution of erythrosine is used as plaque disclosing agent in dental clinics[3,4].

The antimicrobial activity of erythrosine has been demonstrated in previous studies. Begue *et al*.[3] reported that 3% aqueous solution of erythrosine inhibited several microorganisms and may cause changes in microbial flora in oral cavities. Baab *et al*.[5] reported that erythrosine inhibited the growth of some oral bacteria at concentrations higher than 0.25%.

In the dental plaque, bacteria are encapsulated in the oral biofilm and have resistance to antibiotics[6]. As erythrosine is widely used for the treatment of dental plaques, confirming susceptibility values of oral biofilm bacteria as well as planktonic bacteria to erythrosine is necessary. For planktonic bacteria, the commonly used method for detecting microbial

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susceptibility to antimicrobials is to measure the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For biofilms, the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were proposed as experimental benchmarks previously[7]. MBIC of an antimicrobial agent is the minimum concentration at which the growth of biofilm bacteria is inhibited, while MBEC is the minimum concentration at which 99.9% of biofilm bacteria are eradicated[7-9]. The aim of this study was to investigate the antimicrobial effect of erythrosine on *Mutans streptococci* by determining MIC, MBC, MBIC, and MBEC values.

Ⅱ. Materials and Methods

1. Bacterial strain and culture conditions

This study employed the *S. mutans* ATCC 25175 (KN88) strain. Bacteria were incubated in brain-heart infusion broth (BHI; Becton, Dickinson, Franklin Lakes, NJ) at 37°C under aerobic conditions with 5% CO_2 for 18 h. Suspension turbidity was measured by a spectrophotometer (Smart Plus 2700; Youngwoo inst., Seoul, Korea). A standard curve of culture turbidity and bacterial cell numbers was established and utilized for cell dilutions. The bacteria were diluted to 10^7 colony-forming units (CFU)/mL with phosphate-buffered saline (PBS) to determine MIC and MBC and diluted to 10^8 CFU/mL with PBS to determine MBIC and MBEC.

2. Antimicrobial agent

A solution of 10000 μg/L (10% w/v) erythrosine (Sigma-Aldrich, St Louis, MO) was prepared in PBS. This solution was filtered-sterilized and stored at 4°C in the dark. The solution was then diluted in BHI broth at a concentration range of 0.02 - 10000 μg/L.

3. Determination of MIC and MBC

To investigate the antimicrobial activity of erythrosine, the MIC was measured by the microdilution method. The susceptibility test was conducted according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The bacterial suspension was serially diluted in 96-well plates (SPL Life Sciences, Pocheon, Korea) using BHI medium. After incubation at 37°C for 18 h in a 5% CO_2 incubator, the minimum concentration at which bacterial growth was inhibited was determined as the MIC. The culture broths of wells with higher MICs were plated on blood agar plates (Hangang, Gunpo, Korea), and the concentration at which 99.9% of bacteria were killed was determined as the MBC. If the same results were obtained in 2 repeated experiments, the values were set as MIC and MBC values. If experiment results were different, the tests were repeated to determine the MIC and MBC.

4. Determination of MBIC and MBEC

To form *S. mutans* biofilms on the bottom of 96-well plates (Flat-Bottom Plate; SPL Life Sciences), 180 μL BHI medium and 20 μL bacterial suspension (10^8 CFU/mL) were added to the plates and cultured in a 5% CO_2 incubator at 37°C for 18 h. After incubation, each microplate was washed 3 times with BHI medium to remove any non-adherent bacterial cells. Erythrosine was serially diluted in BHI medium in a new microplate and then the diluted solution was transferred to the microplates with biofilms, which were then cultured at 37°C for 18 h. After incubation, supernatant turbidity was examined and the minimum concentration at which bacterial growth was inhibited was determined as the MBIC. After MBIC determination, the erythrosine-containing microbial culture was washed 3 times with BHI medium. Then, fresh BHI culture medium without erythrosine was added to the microplates and incubation was carried out for 18 h at 37°C. Supernatant turbidity of this biofilm culture was observed visually to determine the minimum concentration at which the bacteria no longer grew, which was designated the MBEC. All experiments were repeated twice, and if the results were inconsistent, the experiment was repeated until consistent MBIC and MBEC values were obtained.

Ⅲ. Results

1. MIC and MBC of erythrosine

Erythrosine MIC and MBC against *S. mutans* in the planktonic state are shown in Table 1. The determined MIC and MBC values after repeated experiments were both 19.5 μg/L.

2. MBIC and MBEC of erythrosine

Erythrosine MBIC and MBEC against *S. mutans* biofilms are

Table 1. Antibacterial activity of erythrosine against planktonic and biofilm *Mutans streptococci*

	Planktonic		Biofilm	
	MIC	MBC	MBIC	MBEC
Erythrosine (μg/L)	19.5	19.5	313	2500

shown in Table 1. The determined MBIC and MBEC values after repeated experiments were 313 μg/L and 2500 μg/L, respectively.

IV. Discussion

The antibacterial effect of erythrosine on planktonic *S. mutans* was verified in a previous study. Baab *et al*.[5] demonstrated that erythrosine inhibited the growth of *S. mutans* through an agar-dilution susceptibility test and reported 0.13% erythrosine as the MIC. Despite using the same *S. mutans* strain (ATCC 25175), the previously reported value was approximately 6-fold higher than our MIC result. The reason for this discrepancy is thought to be the differences in culture conditions of the microorganism and the experimental method. In our study, BHI liquid medium and the microdilution method were used, compared with blood-agar solid medium and the agar-dilution test employed in the previous study[5]. The antimicrobial effect of erythrosine is thought to be due to 4 iodine radicals in its molecular structure[5,10].

In this study, the determined MIC and MBC values were the same. According to this result, erythrosine appeared to kill 99.9% of planktonic *S. mutans* at the MIC concentration and thus has a bactericidal rather than a bacteriostatic effect. To biofilm, erythrosine showed antibacterial effects at higher concentrations than those used for planktonic bacteria. This result is consistent with previous studies reported that the MIC value of an antimicrobial agent cannot be applied to effectively treat the biofilm[7].

The acute oral toxicity (lethal dose 50) of erythrosine is 1264 mg/kg for mice and 1840 mg/kg for rats[2]. In this study, *S. mutans* biofilm growth was inhibited at erythrosine concentrations lower than those clinically used in dental offices as well as the dose that leads to systemic side effects. Based on this result, further experiments should be performed to evaluate the effect of erythrosine on dental plaque at clinically used concentrations in dental office.

V. Conclusion

In the present study, the minimum susceptibility values of erythrosine against *S. mutans* in planktonic and biofilm states were measured. For planktonic bacteria, erythrosine exhibited a bactericidal effect at 19.5 μg/L. For biofilms, higher doses of erythrosine were necessary for its bacteriostatic (313 μg/L MBIC) and bactericidal effects (2500 μg/L MBEC).

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국문초록

부유 상태와 바이오필름 상태에서 *Mutans streptococci*의 Erythrosine에 대한 감수성 평가

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이 연구의 목적은 부유 및 바이오필름 상태의 *Mutans streptococci*에서 치태와 치석 착색제로 치과 진료실에서 사용하는 식용색소인 에리스로신에 대한 감수성을 조사하는 것이다.

세균은 호기성 조건에 배양하였고, 에리스로신은 0.02 - 10000 μg/L의 농도로 희석하였다. 부유 상태 *S. mutans*의 최소 억제 농도(MIC)와 최소 살균 농도(MBC)를 측정하였다. 세균을 배양하여 바이오필름을 형성한 후, 최소 바이오필름 억제 농도(MBIC) 및 최소 바이오필름 박멸 농도(MBEC)를 측정하였다.

MIC와 MBC는 19.5 μg/L로 동일하게 측정되었으며 따라서 에리스로신은 부유 상태의 *S. mutans*에 대해 살균 효과를 가지는 것으로 나타났다. MBIC, MBEC 값은 각각 313 μg/L, 2500 μg/L로 측정되었다. 측정된 MIC, MBC, MBIC는 실제로 치과에서 사용되는 농도에 비해 낮은 값이며 따라서 인체에 무해한 농도에서 *S. mutans*를 억제할 수 있을 것으로 보인다.