Efficacy of Disinfectants against Naturally Occurring and Artificially Cultivated Bacteria

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Naturally occurring bacteria, is exist in nature, and is never cultivated on conventional culture medium. We evaluated the efficacy of disinfectants against naturally occurring bacteria in in-use cotton balls soaked in 0.02% benzalkonium chloride solution which had been used to disinfect the genital area by patients undergoing self-catheterization at home and the same bacteria subcultured on nutrient broth (artificially cultivated bacteria). The colony forming units (CFU) of naturally occurring bacteria such as *Serratia marcescens*, *Alcaligenes xylosoxidans*, and *Burkholderia cepacia* were not decreased after 48 h exposure to 0.025–0.1% benzalkonium chloride solution, but the same strains subcultured on nutrient broth were killed within only 10 min exposure to 0.025–0.1% benzalkonium chloride solution. In addition, the CFU of these three kinds of naturally occurring bacteria were not decreased after 48 h exposure to 0.02% chlorhexidine gluconate solution, but the same strains subcultured on nutrient broth were killed within 2 h exposure to chlorhexidine gluconate solution. The result showed that disinfectant efficacy differed markedly against naturally occurring and artificially cultivated bacteria. Therefore, it is preferable to use the naturally occurring bacteria not only artificially cultivated bacteria when examining disinfectant efficacy.

Key words  naturally occurring bacteria; artificially cultivated bacteria; disinfectant; benzalkonium chloride; chlorhexidine gluconate; efficacy

The majority of reports regarding the efficacy of disinfectants on bacteria were from the results obtained in bacteria cultivated with nutrient medium, referred to as artificially cultivated bacteria, and there were few reports from the studies performed using bacteria which have never been cultivated with nutrient medium, referred to as naturally occurring bacteria.1–4) Based on the survey by ourselves, only the following information was obtained. Carson et al. reported that naturally occurring *Pseudomonas aeruginosa* isolated from the distilled water reservoir of a hospital mist therapy unit were markedly more resistant to inactivation by 67 ppm chlorine dioxide, 2.8 ppm quaternary ammonium compound, 0.25% acetic acid, and 31 ppm glutaraldehyde than cells subcultured once on trypticase soy agar.5) In addition, the concentrations of the disinfectants used in this report were below 1/100 of generally used concentrations. Therefore, in the present study, the effects of disinfectants on naturally occurring bacteria and artificially cultivated bacteria were comparatively examined using the disinfectants at the generally used concentrations. In this study, contaminated bacteria from the cotton balls soaked in 0.02% benzalkonium chloride solution, which had been used to disinfect the genital area by patients undergoing self-catheterization at home during 7 d or more were used as the naturally occurring bacteria.

MATERIALS AND METHODS

**Strains and Agents Used** *Serratia marcescens*, *Alcaligenes xylosoxidans*, and *Burkholderia cepacia* in in-use cotton balls soaked in 0.02% benzalkonium chloride solution which had been used to disinfect the genital area by patients undergoing self-catheterization at home during 7 d or more were used as naturally occurring bacteria.6) These three bacteria were identified by Gram staining, morphological examination, the oxidation-fermentation test, the cytochrome-oxidase test, and the API system (Analytab Products, Plainview, New York, U.S.A.). Cotton balls which containing contaminated bacteria were centrifuged at 3000×g for 10 min, and suspended with sterile physiologic saline. Thus bacterial suspension of the naturally occurring bacteria (ca. 10^4–6 colony forming units [CFU/mL]) was obtained. Then, the bacterial suspension of artificially cultivated bacteria was obtained as follows. One tenth milliliters solution from cotton balls soaked in a 0.02% benzalkonium chloride solution which polluted by the each naturally occurring bacteria was inoculated into 10 mL of nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated at 30°C for overnight. The each subculture were re-inoculated into fresh 10 mL of nutrient broth and incubated at 30°C for overnight. The each second subculture were centrifuged at 3000×g for 10 min, and suspended with sterile physiologic saline to obtain about 10^4–6 CFU/mL.

The disinfectants used in this study were 10% benzalkonium chloride solution (Kenei Pharmaceutical Co., Ltd., Osaka, Japan), 5% chlorhexidine gluconate solution (Kenei Pharmaceutical Co., Ltd.), ethanol for disinfection (76.9–81.4% [v/v] ethanol solution, Kenei Pharmaceutical Co., Ltd.), and 1% sodium hypochlorite solution (Kenei Pharmaceutical Co., Ltd.). These disinfectants were diluted with sterilized distilled water as needed. Bovine serum albumin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used in this study as organic matter. Bovine serum albumin was dissolved in sterile distilled water at a concentration of 2 g/L and sterilized by filtration through 0.22 µm pore-size filters.

**Evaluation of Disinfectant Efficacy** At first 0.5 mL of 0.2% bovine serum albumin solution was added to 9 mL of each disinfectant, and 0.5 mL of bacterial suspension was inoculated into 9.5 mL of each disinfectants at 20°C (final concentration of bovine serum albumin was 0.01%). After standing for ten minutes and 1, 2, 24, and 48 h, 1 mL aliquot of

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the suspension were collected and added to 9 mL of solution containing 0.5% lecithin (Wako Pure Chemical Industries, Ltd.) and 1.0% Lubrol W (Nikko Chemicals Co., Ltd., Tokyo, Japan) as inactivators. Ethanol for disinfection was inactivated by diluting it 1:100 with solution containing 0.5% lecithin and 1.0% Lubrol W. One tenth percents of sodium thiosulfate (Wako Pure Chemical Industries, Ltd.) was used as inactivators when 0.05% sodium hypochlorite solution was examined. In addition, we also carried out the examination which used sterile physiologic saline instead of the disinfectant as control. Suspensions were serially diluted ten-fold with sterile physiologic saline. The undiluted or diluted samples (0.2 mL) were plated on nutrient agar (Eiken Chemicals Co., Ltd.), streaked with a plastic “hockey stick” and incubated at 30°C for 48 h to count viable cells.

Statistical Analysis Cell numbers as CFU/mL were transformed to the log_{10} CFU/mL. The survivors value after exposure to each disinfectants were compared with the initial value using one-way ANOVA. Post hoc multiple comparisons were made using the Scheffe’s F test, and p<0.05 was considered statistically significant.

Morphologic Scanning Electron Microscopy Each bacterial culture was suspended in phosphate-buffered saline (PBS) and then placed on a carbon-deposited coverglass. After 10 min, the coverglass was immersed in 1% glutaraldehyde (Wako Pure Chemical Industries, Ltd.) in PBS for more than 10 min, the coverglass was immersed in 1% glutaraldehyde (PBS) and then placed on a carbon-deposited coverglass. After drying, the bacterial culture was suspended in phosphate-buffered saline. The solution was dried. After applying a platinum coating to the coverglass, scanning electron microscope (S 890, JEOL Ltd., Tokyo, Japan) observations were performed.

RESULTS

Efficacy of Disinfectants against Naturally Occurring and Artificially Cultivated Bacteria The CFU of naturally occurring S. marcescens were not decreased after 48 h exposure to 0.02% benzalkonium chloride solution, but artificially cultivated S. marcescens were killed within 10 min (Fig. 1 left). In addition, the CFU of naturally occurring S. marcescens were not decreased after 48 h exposure to 0.02% chlorhexidine gluconate solution, furthermore 0.02% and 0.05% chlorhexidine gluconate solution were needed 2–24 h to kill them. However, artificially cultivated S. marcescens were killed within 1 h after exposure to 0.02–0.1% chlorhexidine gluconate solution (Fig. 1 right).

The CFU of naturally occurring A. xylosoxidans were decreased after 2 h exposure to 0.1% benzalkonium chloride solution, but multiplied after 24 h. On the other hand, 0.025% and 0.05% benzalkonium chloride solution were not decreased them. However, artificially cultivated A. xylosoxidans were killed within 10 min (Fig. 2 left). In addition, the CFU of naturally occurring A. xylosoxidans were not decreased after 48 h exposure to 0.02% chlorhexidine gluconate solution, and were killed within 24 or 48 h exposure to 0.1% or 0.05% chlorhexidine gluconate solution, respectively. However, artificially cultivated A. xylosoxidans were killed within 10 min after exposure to 0.02–0.1% chlorhexidine gluconate solution (Fig. 2 right).

The CFU of naturally occurring B. cepacia did not show any decrease even after 48 h exposure to 0.025–0.1% benzalkonium chloride solution, but artificially cultivated B. cepacia were killed within 10 min (Fig. 3 left). Furthermore, 0.02–0.1% of chlorhexidine gluconate did not have an effect at all against the CFU of naturally occurring B. cepacia. However, artificially cultivated B. cepacia were killed within 2 h exposure to 0.02–0.1% chlorhexidine gluconate solution (Fig. 3 right).

The three strains of naturally occurring or artificially cultivated bacteria were killed within 10 min exposure to ethanol for disinfection and 0.05% sodium hypochlorite solution. In addition, the CFU of the three strains of naturally occurring or artificially cultivated bacteria were increased about 10-fold after 48 h in sterile physiologic saline.

Morphologic Scanning Electron Microscopy Observations Naturally occurring S. marcescens was smaller in size and had fewer flagella compared with its artificially cultivated counterpart (Fig. 4). The same features were also observed in A. xylosoxidans and B. cepacia (data not shown).

DISCUSSION

When bacterial contamination for the cotton balls soaked in 0.02% benzalkonium chloride solution, which are used in...
self-urethral catheterized outpatients at the urology department of one university hospital, was investigated, 20 of 30 samples tested (66.7%) were contaminated with bacteria of $10^3–10^8$ CFU/mL of squeezed solution. Therefore, the effects of the following disinfectants on these bacteria were examined using these contaminated bacteria as the naturally occurring bacteria and these naturally occurring bacteria cultured with nutrient broth as the artificially cultivated bacteria. The disinfectants used were benzalkonium chloride, chlorhexidine gluconate, sodium hypochloride and alcohol, either of which are commonly used in the hospital. The results revealed that the effects of benzalkonium chloride and chlorhexidine gluconate were different largely between the naturally occurring bacteria and artificially cultivated bacteria; either of the disinfectants were less effective on the naturally occurring bacteria and more effective on the artificially cultivated bacteria. The majority of efficacy judgments for disinfectants are usually performed using artificially cultivated bacteria, but, it was proved that the practical efficacy of disinfectants was difficult to be discussed in the experiment using such artificially cultivated bacteria.

It has already been clarified that biofilm-formed bacteria cells are more resistant to disinfectants compared to planktonic cells without Biofilm. Naturally occurring bacteria were showed resistance to disinfectants, but in electron microscopy observations, no biofilm formation was observed in either of $S. marcescens$, $A. xylosoxidans$, and $B. cepacia$ used in the present study. Morphologic scanning electron microscopy revealed that naturally occurring bacteria were small in size and had few flagella, as reported previously. It is guessed that these morphologic differences are caused by proliferated condition of naturally occurring bacteria were under limitation of nutrition such as cotton balls soaked in 0.02% benzalkonium chloride solution.

Alcohol and sodium hypochloride showed excellent...
disinfectant effects on *S. marcescens*, *A. xylosoxidans*, and *B. cepacia* used as the naturally occurring bacteria in the present study. Therefore, alcohol and sodium hypochloride are recommended to be used for disinfection of these bacteria resistant to benzalkonium chloride and chlorhexidine gluconate.

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