J. Pharm. Pharmacol. 2000, 52: 1547–1552
Received March 3, 2000
Accepted July 26, 2000

Electron-microscopic Study of the Bactericidal Effect of OPB-2045, a New Disinfectant Produced from Biguanide Group Compounds, Against Methicillin-resistant *Staphylococcus aureus*

YOSHIKAZU SAKAGAMI, KEIJI KAJIMURA AND HIROSHI NISHIMURA

Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan

Abstract

The bactericidal effect of OPB-2045, a new disinfectant produced from biguanide group compounds, against methicillin-resistant *Staphylococcus aureus* (MRSA), MRSA IID 1677, was investigated by transmission electron microscopy.

OPB-2045 showed strong bactericidal activity against MRSA. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of OPB-2045 against the test strain were 0·78 and 1·56 $\mu g\,mL^{-1}$, respectively. The test bacteria were incubated in the presence of OPB-2045 at 1/2 MIC (0·39 $\mu g\,mL^{-1}$), 1 MIC (0·78 $\mu g\,mL^{-1}$), 2 MIC (1 MBC, 1·56 $\mu g\,mL^{-1}$), 4 MIC (2 MBC, 3·13 $\mu g\,mL^{-1}$) or 10 MIC (5 MBC, 7·8 $\mu g\,mL^{-1}$) at 37°C for 30 s, 3 min, 30 min or 6 h. The morphology of the cells was examined by transmission electron microscopy.

The cell damage observed after 30-min or 6-h incubation in the presence of OPB-2045 at 1/2 or 1 MIC was the same as that at 2, 4 or 10 MIC. The numbers of damaged MRSA cells increased according to the increase in concentration of added disinfectant, and the image of bacteriolysis was observed, too.

After treatment at 1/2 or 1 MIC, a few leaking cells were recognized, but no destroyed cells were found. No morphological changes were observed after treatment at 1 or 2 MIC for 30 s, 3 min or 30 min. When the incubation time was extended to 6 h, morphological changes in the MRSA cells treated at 1 or 2 MIC were observed.

When examining the relationship between the numbers of surviving bacteria and the MIC (MBC) values in soybean casein digest broth, no decrease in MRSA cell numbers was recognized in the untreated control or at 1/2 MIC, but a marked decrease in MRSA cell numbers was recognized as the OPB-2045 concentration was increased.

The new disinfectant OPB-2045 would make a useful contribution to the medical field for the prevention of infections caused by pathogenic bacteria such as MRSA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) are major human pathogens in hospitals worldwide (Crossley 1998; Lowy 1998; Fluckiger & Widmer 1999). Many trials have been performed concerning the prevention of MRSA and VRE infections, but further trials are necessary for the eradication of nosocomial infections.

Disinfectants have been used widely for the prevention of infection by bacteria such as *Pseudomonas aeruginosa*, MRSA and VRE. Recently, some structural and formulation modifications of disinfectants have been performed, and preparations of commercially available disinfectants with minor changes have appeared on the market, but it is desirable to produce a new disinfectant.

OPB-2045, a new disinfectant, is a compound containing a biguanide active base. Sakagami et al (1999) investigated the bactericidal effect of OPB-2045 against *P. aeruginosa* by electron microscopy, and demonstrated its good bactericidal performance.

In this study, we have investigated the bactericidal activity of OPB-2045 against MRSA, as a representative of Gram-positive bacteria, by electron microscopy.

Correspondence: Y. Sakagami, Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan.
E-Mail: sakagami@iph.pref.osaka.jp

Materials and Methods

Bacteria
S. aureus IID 1677 (MRSA IID 1677), purchased from the Institute of Medical Science, The University of Tokyo, was used in this study.

Medium
Soybean casein digest (SCD) broth (Nihon Pharm. Co., Ltd) was used for pre-incubation of the test bacteria and for the experiment of the killing curve of MRSA. Mueller-Hinton medium (DIFCO) was used for measurement of minimum inhibitory concentration (MIC). Mueller-Hinton broth was used for measurement of minimum bactericidal concentration (MBC).

Disinfectant
OPB-2045 (1-(3,4-dichlorobenzyl)-5-octylbiguanide monohydrochloride hemihydrate), a new disinfectant produced from biguanide group compounds (Otsuka Pharm. Co., Ltd), was used in this study.

MIC and MBC
The MIC of OPB-2045 against MRSA was measured by the agar dilution method (Goto et al 1981) using micro-planters (Sakuma Seisakusho Co., Ltd, Japan). The MBC of OPB-2045 against MRSA was measured by the method of Bearbeiter von Beck et al (1977).

Transmission electron microscopy
Transmission electron microscopy was performed by the method of Hayat (1986). A broth culture of MRSA was centrifuged at 2500 rev min^{-1} for 15 min, and the bulk of the supernatant was removed, and the cell pellet was washed twice with sterile physiological saline. Test sample solution (2 mL) at the designated concentration (control, 0·39 (1/2 MIC), 0·78 (1 MIC), 1·56 (2 MIC), 3·13 (4 MIC) or 7·8 $\mu g\,mL^{-1}$ (10 MIC)) was added, and the mixtures were incubated at 37°C for 30 s, 3 min, 30 min or 6 h. After centrifugation (2500 rev min^{-1} for 15 min), 5 mL modified Karnovsky solution (Karnovsky 1965) (a mixture of 1% paraformaldehyde and 1% glutaraldehyde) was added, and the samples were incubated for 2 h at 20°C. After removal of the Karnovsky solution, the samples were further processed for transmission electron microscopy by routine methods, and the morphology of the cells was examined.

Relationship between the numbers of surviving MRSA cells and the MIC (or MBC) values
OPB-2045 was diluted in distilled water or SCD broth, and 10-mL test sample solution at each concentration (control, 0·39 (1/2 MIC), 0·78 (1 MIC), 1·56 (2 MIC), 3·13 (4 MIC), 7·8 (10 MIC) or 15·6 $\mu g\,mL^{-1}$ (20 MIC)) was prepared. The appropriate volume of MRSA cell suspension (20-h pre-incubation at 37°C) was added (final bacterial count: approximately $2{\cdot}0\times10^{6}$ cells mL^{-1}), and the cells were incubated at 37°C for 30 s, 3 min, 30 min or 6 h. Bacterial cell numbers in each test tube were determined by the agar dilution method using SCD agar (Nihon Pharm. Co., Ltd).

Results

MIC and MBC values of OPB-2045 against MRSA
The MIC and MBC values of OPB-2045 against MRSA IID 1677 were 0·78 and 1·56 $\mu g\,mL^{-1}$, respectively.

Bactericidal effect of OPB-2045 as observed by transmission electron microscopy
Figures 1–8 demonstrate the bactericidal effect of OPB-2045 as observed by transmission electron microscopy.
Observation of ultra-thin sections found no change in cells of the untreated control group during the 30-s or 6-h incubation periods with respect to cell components, the cell membrane or the cell wall (Figures 1 and 2).

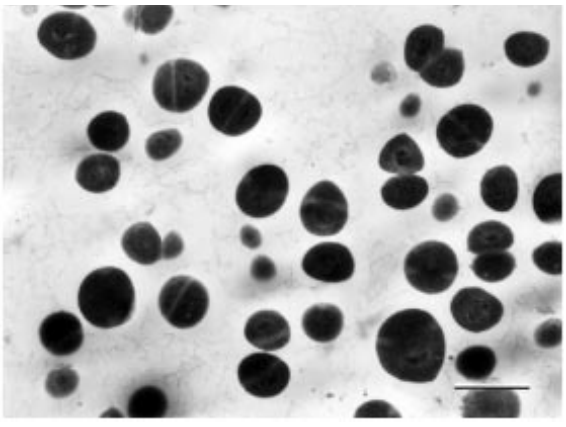

Figure 1. Transmission electron micrograph of an ultra-thin section showing the morphology of MRSA cells in the control incubated for 30 s in the absence of OPB-2045 (×10 000, scale 1 μm).

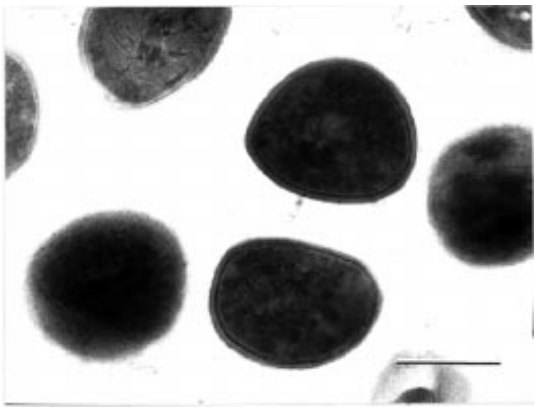

Figure 2. Transmission electron micrograph of an ultra-thin section showing the morphology of MRSA cells in the control incubated for 6 h in the absence of OPB-2045 (×40 000, scale 0·4 μm).

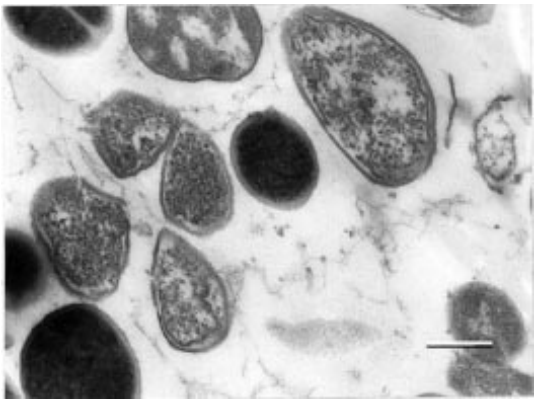

Figure 5. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 7·8 μg mL^{-1} for 3 min at 37°C (×25 000, scale 0·5 μm).

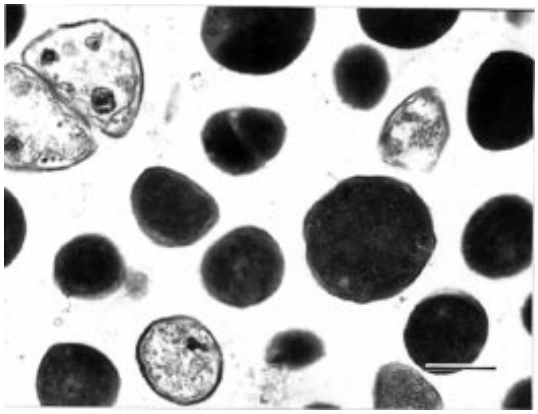

Figure 3. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 7·8 μg mL^{-1} for 30 s at 37°C (×20 000, scale 0·5 μm).

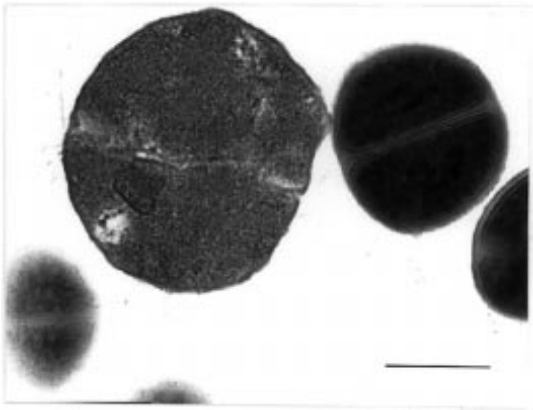

Figure 6. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 1·56 μg mL^{-1} for 30 min at 37°C (×40 000, scale 0·5 μm).

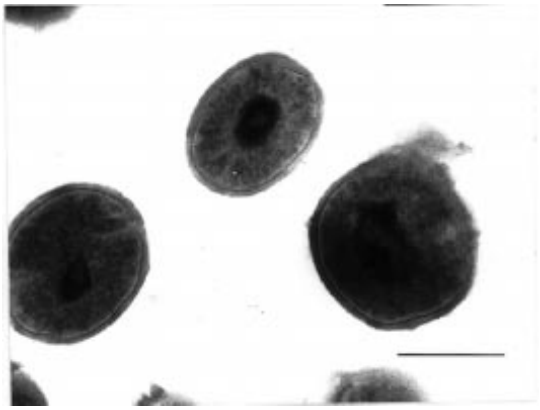

Figure 4. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 3·13 μg mL^{-1} for 30 s at 37°C (×40 000, scale 0·5 μm).

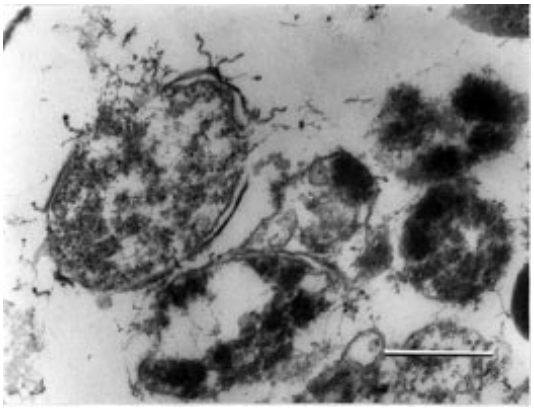

Figure 7. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 7·8 μg mL^{-1} for 6 h at 37°C (×40 000, scale 0·5 μm).

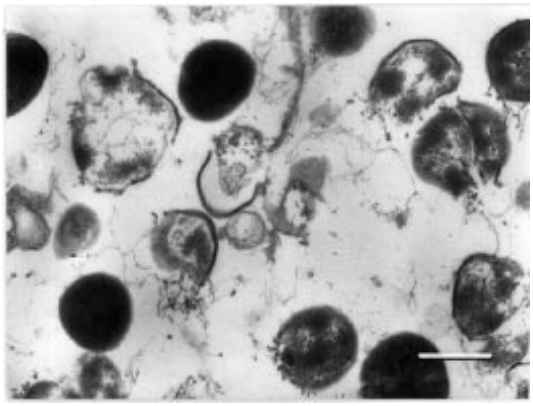

Figure 8. Transmission electron micrograph of an ultra-thin section showing the bactericidal effect of OPB-2045 against MRSA cells treated with OPB-2045 at 0·78 μg mL^{-1} for 6 h at 37°C (×20 000, scale 0·5 μm).

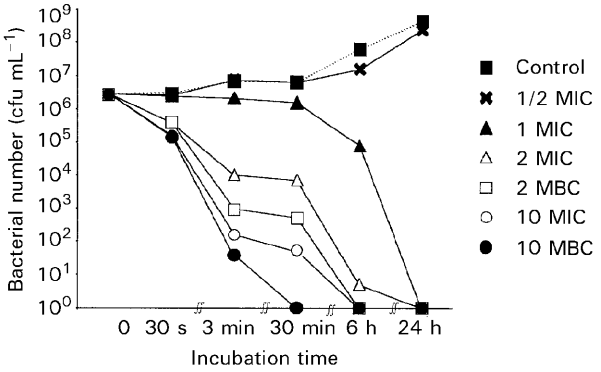

Figure 9. Relationship between the numbers of surviving MRSA in SCD broth and the MIC (or MBC) values.

After treatment of the cells with OPB-2045 for 30 s at 37°C, empty cells were observed at 10 MIC (7·8 μg mL^{-1}) (Figure 3). Some damaged cells were also observed at 4 MIC (3·13 μg mL^{-1}) (Figure 4), whereas no cell damage was observed at concentrations of OPB-2045 below 2 MIC (1·56 μg mL^{-1}).

After treatment of the cells with OPB-2045 for 3 min at 37°C, increased numbers of destroyed cells were observed at 4 MIC and at 10 MIC (Figure 5). Some damaged cells were observed at 2 MIC (1·56 μg mL^{-1}), but not at concentrations below 1 MIC (0·78 μg mL^{-1}).

After treatment for 30 min at 37°C, some damaged cells were observed at 1 and 2 MIC (Figure 6), but not at concentrations below 1/2 MIC (0·39 μg mL^{-1}).

After treatment for 6 h at 37°C, increased numbers of damaged cells were observed at 4 (data not shown) and 10 MIC (Figure 7). Some leaking cells were observed at 1 MIC (Figure 8), and some damaged cells were also recognized at 1/2 MIC.

It is evident that when the incubation time was extended, the degree of damage to MRSA increased at a low concentration of OPB-2045 (1 MIC). With respect to the degree of morphological damage against MRSA, the numbers of leaking and destroyed cells increased in accordance with the increase in concentration of added OPB-2045. After treatment of the cells with a low concentration of OPB-2045, no destroyed cells were observed except for some empty cells.

When the incubation time was extended, morphological damage to MRSA was observed at 1 and 2 MIC, at which concentrations no damage was recognized after incubation for 30 s or 3 min at 37°C.

Relationship between the numbers of surviving bacterial cells and the MIC (or MBC) values

In distilled water, the numbers of MRSA cells in the control group did not decrease in the 30-s, 3-min, 6-h or 24-h incubation period. In the test group with OPB-2045 added (at 1/2 MIC, 1 MIC, 2 MIC, 2 MBC, 10 MIC or 10 MBC), dissolved in distilled water, no viable bacterial cells were found after 30-s, 3-min, 30-min, 6-h or 24-h incubation (data not shown).

When OPB-2045 was dissolved in SCD broth, increased numbers of MRSA cells were observed in the control group and in the 1/2 MIC test group after 6-h and 24-h incubation, but a marked decrease in bacterial numbers was recognized at 1 MIC, 2 MIC, 4 MIC, 10 MIC and 10 MBC (Figure 9).

Discussion

MRSA is an important cause of nosocomial infections and has become endemic in hospitals worldwide. Control of MRSA infection and colonization is difficult and may result in serious clinical and managerial problems (Cohen 1992; de Lancastre et al 1994; Dominguez et al 1994; Teixeira et al 1995). Recently, many trials have been performed concerning prevention of the occurrence of nosocomial infections caused by bacteria such as MRSA and VRE. However, further trials are necessary to reduce the incidence of such infections to a very low level.

Disinfectants are mainly used as the means of prevention of the occurrence of nosocomial bacterial infections. However, none of those trials had led to the development of a new disinfectant. In our opinion, for prevention of bacterial infections it is necessary to supply a new disinfectant for use in

the medical field. Thus, we have studied the bactericidal effect of OPB-2045 against MRSA by transmission electron microscopy. Other studies on the bactericidal effects of disinfectants by electron microscopy have been performed (Arimura & Kitagawa 1973; Richards & Cavill 1976, 1979a, b; Gelinas & Goulet 1983; Bobichon & Bouchet 1987). Electron-microscopic studies have a good impact, allowing us to understand the bactericidal activity of a disinfectant. Transmission electron microscopy is usually used to observe the cell damage induced, such as the empty cells or the leaking cells.

The MIC and MBC values of OPB-2045 against MRSA IID 1677 were 0·78 and 1·56 $\mu g\,mL^{-1}$, respectively. These findings suggest that OPB-2045 has strong antibacterial activity.

To clarify the mechanism of action of OPB-2045 against MRSA the morphological changes in MRSA cells after 30-s, 3-min, 30-min and 6-h incubation at 1/2, 1, 2 and 10 MIC were examined. No cell damage was found in the untreated control group after incubation at 37°C for 30 s or 6 h. However, in the OPB-2045-treated groups incubated at 37°C for 30 min or 6 h, increased numbers of empty cells, from which the cell components had leaked, were observed. According to the increase in concentration of added OPB-2045, the numbers of empty cells increased, markedly.

After treatment of the cells at a low concentration of OPB-2045, no destroyed cells were observed except for some empty cells. According to the increase in incubation time in the presence of OPB-2045, the damage to MRSA became great at low concentration (1 MIC). With respect to the degree of the morphological effect against MRSA, the numbers of leaking and destroyed cells increased in accordance with the increase in concentration of added OPB-2045.

When the incubation time was extended, morphological damage to MRSA cells was observed at 1 and 2 MIC, at which concentrations no damage was recognized after incubation for 30 s or 3 min at 37°C. These results suggest that OPB-2045 may act on the cell wall of MRSA, and would destroy them when present at a concentration of 1 MIC (1 MBC).

When examining the relationship between the numbers of surviving bacteria and the MIC (or MBC) values in SCD broth, a decrease in bacterial numbers was recognized according to the increase in concentration of added OPB-2045 above 1 MIC (0·78 $\mu g\,mL^{-1}$).

OPB-2045 showed suitable bactericidal action against the MRSA tested and would be useful as a disinfectant. However, particular attention for use under dirty conditions would be necessary, as we have found that its bactericidal activity is diminished in the presence of organic material (data not shown).

Previously we investigated the mechanism of the bactericidal effect of OPB-2045 against *P. aeruginosa*, a Gram-negative rod, by transmission and scanning electron microscopy (Sakagami et al 1999). We think that good results were obtained at both high and low magnification. Few electron microscopy studies examining morphological changes in MRSA have been reported (Hamilton-Miller & Shah 1999). Our report on the bactericidal activity of OPB-2045 may be valuable in terms of research using the transmission electron microscope.

References

Arimura, H., Kitagawa, T. (1973) Electron microscope studies on the effect of bronopol (2-bromo-2-nitro-1,3-propanediol) on *Pseudomonas aeruginosa*. Chemotherapy 21: 1283–1287

Bearbeiter von Beck, E. G., Borneff, J., Grun, L., Gundermann, K. O., Lanz, E., Lammers, T., Mulhens, K., Primavesi, C. A., Schmidt, B., Schubert, R., Weinhold, E., Werner, H. P. (1977) Recommendations for the test evaluation of the efficacy of chemical disinfectant procedures. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Originale B 165: 335–380

Bobichon, H., Bouchet, P. (1987) Action of chlorhexidine on budding *Candida albicans*: scanning and transmission electron microscopic study. Mycopathologia 100: 27–35

Cohen, M. L. (1992) Epidemiology of drug resistance: implications for a post-antimicrobial era. Science 257: 1050–1055

Crossley, K. (1998) Vancomycin-resistant enterococci in long-term-care facilities. Infect. Control Hosp. Epidemiol. 19: 521–525

de Lancastre, H., Couto, I., Santos, I., Melo-Cristino, J., Torres-Psreira, A., Tomasz, A. (1994) Methicillin-resistant *Staphylococcus aureus* disease in a Portuguese hospital: characterization of clonal types by a combination of DNA typing method. Eur. J. Clin. Microbiol. Infect. Dis. 13: 64–73

Dominguez, M. A., de Lancastre, H., Linares, J., Tomasz, A. (1994) Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. J. Clin. Microbiol. 32: 2081–2087

Fluckiger, V., Widmer, A. F. (1999) Epidemiology of methicillin-resistant *Staphylococcus aureus*. Chemotherapy 45: 121–134

Gelinas, P., Goulet, J. (1983) Efficacy of 8 disinfectants on 3 types of surfaces contaminated by *Pseudomonas aeruginosa*. Can. J. Microbiol. 29: 1715–1730

Goto, S., Yo, K., Kawakita, R., Kosakai, N., Mitsuhashi, S., Nishino, T., Ohsawa, N., Tanami, H. (1981) Re-revision of the method of Minimum Inhibitory Concentration (MIC). Chemotherapy 29: 76–79

Hamilton-Miller, J. M. T., Shah, S. (1999) Disorganization of cell division of methicillin-resistant *Staphylococcus aureus* by a component of tea (*Camellia sinensis*): a study by electron microscopy. FEMS Microbiol. Lett. 176: 463–469

Hayat, M.A. (Japanese edition) (1986) Basic Techniques for Transmission Electron Microscopy. Academic Press Inc., London, pp 2–26, 48–125, 156–172

Karnovsky, M. J. (1965) A formaldehyde-glutaraldehyde fixation of high osmolarity for use in electron microscopy. J. Cell Biol. 27: 137A–138A

Lowy, F. D. (1998) *Staphylococcus aureus* infections. N. Engl. J. Med. 339: 520–532

Richards, R. M. E., Cavill, R. H. (1976) Electron microscope study of the effect of benzalkonium chloride and edetate disodium on the cell envelope of *Pseudomonas aeruginosa*. J. Pharm. Sci. 65: 76–80

Richards, R. M. E., Cavill, R. H. (1979a) Electron-microscope study of the effect of chlorhexidine on *Pseudomonas aeruginosa*. Microbios 26: 85–93

Richards, R. M. E., Cavill, R. H. (1979b) Electron-microscope study of the effect of benzalkonium, chlorhexidine and polymyxin on *Pseudomonas cepacia*. Microbios 29: 23–31

Sakagami, Y., Mimura, M., Kajimura, K., Yokoyama, H., Nishimura, H. (1999) Electron-microscopic study of the bactericidal effect of OPB-2045, a new mono-biguanide disinfectant produced from biguanide group compounds, against *Pseudomonas aeruginosa*. J. Pharm. Pharmacol. 51: 201–206

Teixeira, L. A., Resende, C. A., Ormonde, L. R., Rosenbaum, R., Figueiredo, A. M., de Lencastre, H., Tomasz, A. (1995) Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. J. Clin. Microbiol. 33: 2400–2404