Comparative Fungicidal Activity of a New Quaternary Ammonium Salt, *N*-Alkyl-*N*-2-hydroxyethyl-*N,N*-dimethylammonium Butyl Phosphate and Commonly Used Disinfectants

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Received July 3, 1995; accepted October 25, 1995

We synthesized a new quaternary ammonium salt, *N*-alkyl-*N*-2-hydroxyethyl-*N,N*-dimethylammonium butyl phosphate (ABP) that does not precipitate in the presence of anionic surfactants by incorporating a paired butyl phosphate anion into cationic surfactants. ABP showed much greater bactericidal activities and antirusting effects than benzalkonium chloride (BAC). In this study, the fungicidal effects of ABP were evaluated in comparison with common disinfectants [BAC, chlorhexidine digluconate (CHX) and alkyldiaminoethylglycine hydrochloride (ADE)]. Fungicidal effects were evaluated in 10 strains of 6 fungal species, namely, 3 *Candida albicans*, 2 *Candida tropicalis*, 1 *Candida parapsilosis*, 1 *Aspergillus niger*, 2 *Aspergillus terreus*, and 1 *Trichophyton rubrum*. ABP and BAC showed the same effects on 2 *C. tropicalis* and *C. parapsilosis*, and similar effects on 3 *C. albicans*, with slight differences among the strains. ABP showed quick fungicidal effects against *A. niger* and 2 *A. terreus* of molds in 30 min at 0.1% and in 15 min at 0.2 and 0.4%, but BAC showed slow effects against *A. niger* at 1 h and 2 *A. terreus* at 30 min at 0.2 and 0.4%, respectively. There is one factor which may have caused this difference: the former had a butyl phosphate anion and the latter had a chloride anion as a counter ion. BAC, CHX and ADE needed 24 h to cause notable effects on *A. niger* at 0.1% and 1 h at 0.2%. The new synthetic anti-rust ABP is considered to have sufficient fungicidal effects that are comparable to those of BAC, CHX and ADE.

Key words anti-rust; disinfectant; fungicidal effect; quaternary ammonium salt

Benzalkonium chloride and benzetonium chloride among quaternary ammonium salts are widely used as disinfectants because of their excellent anti-microbial effects and safety.¹⁾ However, they cause rusting in metallic machines and instruments,²⁾ so they are used as mixtures with rust preventatives such as sodium nitrite and dicyclohexyl ammonium nitrite.^{3,4)} Therefore, sodium nitrite was used as an anti-rust agent for dental instruments in Shinshu University Hospital.

These ammonium salts also have the disadvantage of producing water-insoluble precipitates in the presence of anionic surfactants.

We have synthesized some compounds without these disadvantages and evaluated their bactericidal effects and co-solubility in our previous reports.^{5,6)}

In this study, the fungicidal effects of *N*-alkyl-*N*-2-hydroxy-ethyl-*N,N*-dimethylammonium butyl phosphate (ABP), as shown in Table 1, with high anti-microbial effects was compared with three widely used disinfectants against common infection fungi causatives.

MATERIALS AND METHODS

ABP and Commercial Disinfectants The structure of ABP is shown in Table 1. ABP was synthesized as reported elsewhere.^{6,7)}

Benzalkonium chloride (Osvan[®], 10% (w/v), Nihon Pharmaceutical Co., Ltd.; BAC), Chlorhexidine digluconate (Hibitane[®], 20% (w/v), Zeneca Pharmaceutical Co., Ltd.; CHX) and alkyldiaminoethylglycine hydrochloride (Tego51[®], 10% (w/v), Nihon Shoji Co., Ltd.; ADE) were evaluated as reference disinfectants. Diluent with lecithin and Polysorbate 80 Daigo (LP[®] diluent, Wako Chemical Co., Ltd.; LP) was used as an inactivator of ABP, BAC,

CHX and ADE.

Fungi The following fungal strains isolated from clinical materials at the Bacteriological Test Room, Central Testing Division of Shinshu University Hospital, were used.

<i>Candida albicans</i>	3 strains
<i>Candida tropicalis</i>	2 strains
<i>Candida parapsilosis</i>	1 strain
<i>Aspergillus niger</i>	1 strain
<i>Aspergillus terreus</i>	2 strains
<i>Trichophyton rubrum</i>	1 strain

Culture Medium Sabouraud's agar medium (Nissui Pharmaceutical) was used.

Preparation of *Aspergillus* and *Trichophyton* After fungi were cultured in Sabouraud's agar medium at 25 °C for 10 d, 0.01% sodium laurylsulfate-physiologic saline (SLS) was added to this medium, spores were scraped off, and fungal suspensions were prepared for inoculation as shown in Table 2.

Preparation of *Candida* Fungi were cultured in Sabouraud's agar medium at 25 °C for 3 d, suspended in physiologic saline, and prepared for inoculation as

Table 1. Chemical Structures of *N*-Alkyl-*N*-2-hydroxyethyl-*N,N*-dimethylammonium Butyl Phosphate

A mixture containing the monoester phosphate (*a*=1, *b*=2) and diester phosphate (*a*=2, *b*=1) at a molecular ratio of 1:1.

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shown in Table 2.

Measurement of Fungicidal Effects Various disinfectants were diluted into a geometric concentration series (0.1, 0.2, 0.4%), including a concentration for routine use. One milliliter of each fungal suspension prepared in this way was added to 5 ml of each disinfectant solution. These were treated at 25 °C for 15, 30, and 60 min and 24 h, after which 0.1 ml of each mixture was inoculated onto the Sabouraud's agar medium containing 3% LP on three plates for each disinfectant.

The fungicidal effect was evaluated according to the growth of the inoculated fungi of three plates after culturing at 25 °C for 7 d.

Figure 1 show the time required for the disinfectants to completely kill the fungi at each concentration. To study the influence of 0.01% SLS against the compounds, we mixed 5 ml of 0.1% ABP, BAC, CHX and ADE with 0.1 ml of saline. The mixture was used as a control solution. The tests resulted in the compounds being inactivated by 0.01% SLS.

Table 2. Inoculated Doses of Various Fungi

Fungi	Dose (CFU/ml)
<i>Candida albicans</i> A	6.4×10^8
<i>Candida albicans</i> B	4.4×10^8
<i>Candida albicans</i> C	5.7×10^8
<i>Candida tropicalis</i> A	2.2×10^8
<i>Candida tropicalis</i> B	4.5×10^8
<i>Candida parapsilosis</i>	4.8×10^8
<i>Aspergillus niger</i>	3.6×10^7
<i>Aspergillus terreus</i> A	1.3×10^7
<i>Aspergillus terreus</i> B	2.8×10^7
<i>Trichophyton rubrum</i>	5.2×10^7

RESULTS AND DISCUSSION

Recently, pathogenic agents of infection have changed in response to the development of medicine. Fungi, as well as bacteria and viruses, are sometimes caused by opportunistic infection. However, fungal infections are usually hard to diagnose for therapy. Unlike bacteria, fungi are classified into molds that form spores and yeasts, and they are considered to be different in sensitivity to disinfectants.

We synthesized a quaternary ammonium salt-type disinfectant (ABP), which has anti-rust properties and does not produce precipitates, even in the presence of anionic surfactants. This study examined whether or not these disinfectants have sufficient fungicidal effects.

We compared the fungicidal effects of ABP to 3 common disinfectants on 10 fungal strains isolated clinically at our hospital, namely 6 yeast strains of *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* and 4 strains of the molds *Aspergillus niger*, *Aspergillus terreus* and *Trichophyton rubrum*.

Figure 1 shows the fungicidal effects of ABP on various fungal strains. ABP showed sufficient fungicidal effects on 3 *C. albicans* and *C. parapsilosis* in 15 min, and on 2 *C. tropicalis* in 30 min at 0.1%. BAC showed fungicidal effects on 3 *C. albicans*, 2 *C. tropicalis* and *C. parapsilosis* in 15 min at 0.2 and 0.4%.

It killed *A. niger* in 1 h, and 2 *A. terreus* and *T. rubrum* in 30 min at 0.2 and 0.4%, respectively. CHX were weaker than those of BAC, namely, 30 min to 1 h was needed at 0.1% to kill 3 *C. albicans*. It showed fungicidal effects against *A. niger* in 24 h at 0.1%, in 1 h at 0.2%, and in 30 min at 0.4%. It killed 2 *A. terreus* in 30 min at 0.4%. *T. rubrum* was killed by it in 30 min at 0.4 or 0.2% and

Fig. 1. Fungicidal Activities of ABP,^{a)} BAC,^{b)} CHX^{c)} and ADE^{d)}

Concentration of disinfectants: ▨, 0.1%; ▩, 0.2%; ▤, 0.4%. a) ABP, N-alkyl-N-2-hydroxyethyl-N,N-dimethylammonium butyl phosphate. b) CHX, chlorhexidine digluconate. c) BAC, benzalkonium chloride. d) ADE, alkyldiaminoethylglycine hydrochloride.

in 1 h at 0.1%.

ADE killed 2 *C. albicans* in 30 min at 0.1 or 0.2% and in 15 min at 0.4%. It killed 1 *C. albicans* and 2 *C. tropicalis* in 15 min at 0.2 or 0.4%. The effect on *A. niger* was similar to that of BAC; 24 h was needed at 0.1%, and 1 h was needed at 0.2 and 0.4%, indicating slow fungicidal activities.

The results on 2 *A. terreus* were similar to those with *A. niger*. Against *T. rubrum*, its fungicidal effects were observed in 1 h at 0.1% and in 30 min at 0.2 or 0.4%.

A. niger, *A. terreus* and *T. rubrum* strongly resisted BAC, CHX and ADE, more than *C. albicans*, *C. tropicalis* and *C. parapsilosis* did.

However, it was considered that ABP was more effective against the tested fungi than other disinfectants over a short time. In particular, ABP killed *A. niger* and *A. terreus* in 30 min at 0.1% and in 15 min at 0.2 or 0.4%. Its effects on *T. rubrum* appeared in 30 min at 0.1 or 0.2% and 15 min at 0.4%.

Other disinfectants examined showed no sufficient fungicidal effects on *A. niger* unless it was treated at the highest concentration of 0.4% for 30 min to 1 h.

Quick disinfection of this species was previously considered to be impossible.

Generally, molds have been reported to be more resistant to disinfectants than yeasts.⁸⁾ This was also indicated by our results.⁹⁾ The quaternary ammonium salts, BAC and ABP showed the same effects on *C. tropicalis* and *C. parapsilosis* and similar effects on *C. albicans*, despite slight differences among the strains. However, the effects of BAC on *A. niger*, *A. terreus* and *T. rubrum* were different from those have been of ABP.

There are many reports on the bactericidal mechanisms of BAC, but no reports on the fungicidal mechanisms of BAC have been presented.¹⁰⁾ The mode of bactericidal action of BAC is a two-fold action, involving disorganization of the cell membrane followed by denaturation of proteins essential to metabolism and growth.

We assayed the ζ -potential of a bacterial cell and the adsorbability of disinfectants onto synthetic multibilayer-coated piezoelectric crystal, the synthesized anti-rust disinfectant solution, and found that the minimum inhibitory concentration of ABP against *Escherichia coli* was highly correlated to the concentration of ABP at $\zeta=0$ potential, and that ABP are only slightly absorbed; they mostly penetrate through the lipid bilayer.^{11,12)}

BAC is composed mainly of *n*-C₁₂H₂₅ and *n*-C₁₄H₂₉ homologues of the alkyl group. Jono *et al.* reported the effect of the alkyl carbon length on the antibacterial activity of BAC, and C₁₂-BAC is the most effective component.¹³⁾ The chain length of the alkyl group affects the bactericidal activity. And it is considered to also be effective against fungi.

ABP also has an alkyl carbon of C₁₂. Therefore, electrostatic binding of the cationic agents on the cell surface might be affected by alkyl carbon chain length.

There are many factors which might be affected by the characteristics of the cell surface of the microorganisms used, such as the hydrophilicity and lipophilicity of the

compounds.

We synthesized ABP that was an incorporated butyl phosphate anion into a pair anion of a cationic surfactant, while the pair anion of BAC is chloride. Therefore, the effects of ABP might be different from those of BAC against fungi.

The biguanide, CHX, is bacteriostatic in a low concentration to many gram-positive and gram-negative bacteria; much higher concentrations are bactericidal. It also damages the cytoplasmic membrane of yeasts and prevents the outgrowth, but not the germination, of bacterial spores.^{14,15)} In a previous paper, we examined the adsorption and permeation of CHX to *Serratia marcescens*, and suggested that the membranous permeability of the bacteria was related to a specific activity; leakage of a cellular component such as protein.^{16,17)}

In this study, CHX was considered to produce no quick fungicidal effects against *A. niger*, *A. terreus*, and *T. rubrum*.

ADE, an amphoteric surfactant, is widely used as a disinfectant against gram-positive, gram-negative bacteria and *Mycobacterium* species. Also, the effects of ADE against *A. niger*, *A. terreus*, and *T. rubrum* were similar to those of BAC.

From these results, the fungicidal effects of the newly synthesized ABP against various fungi are considered to be sufficiently comparable to those of common disinfectants.

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