Effect of saliva and serum on the adherence of *Candida* species to chlorhexidine-treated denture acrylic

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Summary. The effect of saliva and serum on the adherence of five strains of *Candida albicans* and one each of *C. tropicalis* and *C. glabrata* to chlorhexidine-pretreated acrylic was measured *in vitro*. A four-fold dilution of saliva or serum significantly inactivated the fungicidal effect of chlorhexidine gluconate. Pretreatment of the acrylic with unstimulated mixed saliva for 30 min led to a reduced adherence for all the *Candida* strains tested, whilst a similar pretreatment with serum slightly increased adhesion. Moreover treatment of saliva- or serum-coated acrylic with chlorhexidine gluconate 2% reduced adherence by between 19% and 86%. The inhibition of yeast adherence by chlorhexidine persisted for up to 19 days after the exposure of the acrylic strips to the disinfectant.

Introduction

In chronic atrophic candidosis (CAC), the most prevalent form of oral candidosis (Odds, 1979), yeasts are isolated more often from the fitting surface of the denture than from the palate. The ability of *Candida* species, especially *C. albicans*, to adhere to and colonise denture acrylic is thought to be important in the pathogenesis of this disease. Different species of *Candida* vary in their ability to adhere to denture acrylic *in vitro* and adherence is significantly reduced by pretreatment of the acrylic with chlorhexidine gluconate 2% for 30 min; this regimen was fungicidal for all the strains tested (McCourtie *et al.*, 1985). Serum inactivates the bactericidal action of chlorhexidine *in vitro* (Roberts and Addy, 1981) and it seemed likely that saliva had a similar effect. Therefore it was decided to investigate the effect of chlorhexidine on yeast growth and adherence in the presence of saliva and serum.

Materials and methods

Organisms

Five strains of *C. albicans*, and one each of *C. tropicalis* and *C. glabrata* were used. *C. albicans* MRL 3153 was from the Mycological Reference Laboratory, Central Public Health Laboratories, Colindale, London. *C. albicans* GRI 682 was isolated from a routine cervical smear

from an apparently asymptomatic woman and *C. albicans* strains GDH 2346 and GDH 2023 were from patients with CAC. *C. albicans* GDH 3212 was from saliva from an asymptomatic child, while *C. tropicalis* GDH 1160 and *C. glabrata* GDH 820 were isolated from a patient with CAC and from a tongue lesion respectively. Strains of *C. albicans* isolated from infections were designated I strains and those from symptomless carriers were referred to as C strains, as previously described (McCourtie and Douglas, 1984). All isolates were identified by tests for germ-tube production, sugar-assimilation and fermentation patterns (Lodder, 1970). The organisms were maintained on slopes of Sabouraud Dextrose Agar (Difco) and subcultured monthly. Every 2 months the cultures were replaced by new ones freshly grown from freeze-dried stock.

Growth conditions

The yeasts were grown in Yeast Nitrogen Base Medium (YNB; Difco) containing 500 mM sucrose as previously described (McCourtie and Douglas, 1981). They were harvested after 24 h (stationary growth phase) and washed twice in 0.15 M phosphate-buffered saline (pH 7.2; PBS).

Preparation of acrylic strips

Acrylic strips were prepared as previously described (Samaranayake and MacFarlane, 1980) with the following modifications. Acrylic monomer and polymer (Simplex Rapid; Howmedica International Ltd, 622 Western Avenue, London) were mixed on aluminium foil-covered glass slides and polymerised in a water bath at 40°C. The

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aluminium foil aids recovery of the acrylic from between the slides and the lower temperature for polymerisation reduces bubbling within the acrylic and increases clarity.

Adherence assay

Pools of unstimulated mixed saliva and of serum from several donors were prepared. The saliva was clarified by centrifugation at 17 000 *g* for 30 min at 4°C. To observe the effect of saliva and serum on yeast adherence, acrylic strips were preincubated in clarified saliva or serum for 30 min at room temperature; control strips were similarly preincubated in PBS. Some acrylic strips were further treated with chlorhexidine gluconate (ICI Pharmaceuticals Ltd, Macclesfield) 2% or with sterile distilled water at room temperature for 30 min before performing the adherence assay.

To determine how long acrylic-bound chlorhexidine inhibited yeast adherence, acrylic strips were incubated in chlorhexidine gluconate 2% at room temperature for 30 min and then stored in a moist chamber or submerged in filter-sterilised (Sterifil-D filter unit, Millipore Corporation) clarified saliva until required for adherence assays. Control strips were similarly pretreated with distilled water and stored in the same way. The adherence assay was as previously described (McCourtie and Douglas, 1981). Briefly, transparent acrylic strips were incubated in standardised yeast suspensions (2.5×10^9 cells/ml in PBS), and the number of adherent organisms determined by microscopy. All adherence values quoted were obtained by counting 30 fields on each of duplicate strips from 10 independent assays.

Sensitivity to chlorhexidine gluconate

Fungicidal concentrations of chlorhexidine for the yeast isolates were determined as previously described (McCourtie *et al.*, 1985). To examine the effect of saliva

and serum on the fungicidal concentrations, standardised yeast suspensions (5×10^9 cells/ml in distilled water) were added to 1-ml volumes of various concentrations of chlorhexidine containing saliva or serum at dilutions of 1 in 4, 10 and 20 to yield final yeast concentrations of 2.5×10^9 cells/ml.

Statistical analysis

Student's *t* test was used to evaluate differences in yeast adherence. A *p* value of <0.05 was considered significant.

Results

Effect of saliva and serum on the sensitivity of Candida species to chlorhexidine

The fungicidal concentrations of chlorhexidine gluconate with various concentrations of serum and saliva for the *Candida* species tested are shown in the table. When the assay mixture contained either serum or saliva diluted 1 in 4 there was a marked increase in the fungicidal concentration of chlorhexidine for all the isolates; up to ten times more chlorhexidine was required to achieve the same effect as in the control assays. However, further dilution of serum or saliva reduced its effect and the fungicidal concentrations of chlorhexidine returned to those of control assays when either serum or saliva was diluted 20-fold.

Effect of saliva and serum on yeast adherence

Adherence of all *Candida* strains was reduced by pretreatment of the acrylic with pooled unstimulated mixed saliva for 30 min at room temperature

Table. Effect of saliva and serum on the sensitivity of *Candida* species to chlorhexidine gluconate

Isolate	Fungicidal concentration* of chlorhexidine (% v/v) in						
	Control	Saliva dilution			Serum dilution		
		4	10	20	4	10	20
<i>C. albicans</i> GDH 2023	0.05	0.2	0.05	0.05	0.2	0.2	0.07
<i>C. albicans</i> GDH 2346	0.05	0.1	0.07	0.05	0.1	0.05	0.05
<i>C. albicans</i> MRL 3153	0.05	0.1	0.07	0.05	0.07	0.05	0.05
<i>C. albicans</i> GRI 682	0.1	1.0	0.2	0.2	1.0	1.0	0.5
<i>C. albicans</i> GDH 3212	1.0	2.0	1.0	1.0	1.0	1.0	1.0
<i>C. tropicalis</i> GDH 1160	0.02	0.05	0.05	0.02	0.05	0.05	0.02
<i>C. glabrata</i> GDH 820	0.07	0.1	0.1	0.07	0.1	0.07	0.07

* Organisms were exposed to chlorhexidine for 5 min, and the fungicidal concentration taken as percentage of chlorhexidine in the lowest dilution required to achieve a 100% kill.

Fig. 1. Adherence of *Candida* strains to acrylic pretreated with distilled water (■), saliva (□), or saliva followed by chlorhexidine gluconate 2% (▨). Standard errors were in the range 4.6% to 15.5% of the mean values. A significant decrease in adherence with saliva treatment of acrylic is shown (+) while (Δ) represents significant inhibition of adherence to saliva-coated acrylic by chlorhexidine gluconate 2%. C.a. = *C. albicans*; C.t. = *C. tropicalis*; and C.(g). = *C. glabrata*.

Fig. 2. Adherence of *Candida* strains to acrylic pretreated with distilled water (■), saliva (□), or serum followed by chlorhexidine gluconate 2% (▨). Standard errors were in the range 4.4–13.1% of the mean values. A significant increase in adherence with serum treatment of acrylic is shown (+) while (Δ) represents a significant inhibition of adherence to serum-coated acrylic by chlorhexidine gluconate 2%. C.a. = *C. albicans*; C.t. = *C. tropicalis*; and C.(g). = *C. glabrata*.

(fig. 1). Inhibition of adherence was greater with those strains that adhered to acrylic in greater numbers, i.e., the strains of *C. albicans* isolated from infections (I strain; 34–50%) than with the strains of *C. albicans* from symptomless carriers (C strains) and with *C. tropicalis* GDH 1160 and *C. glabrata* GDH 280 (15–43%). However, inhibition of adherence was not significant for all the other *Candida* isolates (fig. 1). Treatment of the saliva-coated acrylic with chlorhexidine 2% again reduced adherence. Once more inhibition was greatest with the I strains of *C. albicans* (63–75%) than with the C strains of *C. albicans* and with *C. tropicalis* GDH 1160 and *C. glabrata* GDH 820 (19–40%).

Pretreatment of denture acrylic with pooled human serum increased adherence of all *Candida* strains except *C. albicans* GDH 3212 with which adherence was slightly but not significantly reduced (fig. 2). Further treatment of serum-coated acrylic with chlorhexidine 2% inhibited subsequent adherence of all strains of *Candida*. Again, inhibition of adherence to serum-coated acrylic was greatest with I strains of *C. albicans* (83–86%). The significance levels of changes in adherence are given in the caption for fig. 2.

Retention of chlorhexidine by acrylic

Adherence of *C. albicans* GDH 2346 to denture acrylic was inhibited by chlorhexidine gluconate for up to 19 days after treatment with the disinfectant when the acrylic strips were stored in a moist chamber (fig. 3). After this time, the ability of chlorhexidine to reduce yeast adherence was rapidly lost. In an attempt to mimic the conditions in the mouth, the acrylic strips were also stored in filter-sterilised mixed saliva at 37°C. As can be seen from fig. 3, chlorhexidine-treated acrylic incubated in filtered saliva was not as efficient at inhibiting subsequent adherence of *C. albicans* GDH 2346. However inhibition of adherence of 50–60% was maintained for 19 days after initial treatment of the acrylic with chlorhexidine 2%.

Discussion

Trauma to the palatal mucosa is an important aetiological factor in CAC (Nyquist, 1952; Ritchie *et al.*, 1969) and it is likely that any inflammatory serous exudate would coat the fitting surface of the acrylic dentures. Our previous work (Samaranayake *et al.*, 1980; McCourtie and Douglas, 1981)

Fig. 3. Effect of time after pretreatment with chlorhexidine gluconate 2% on the subsequent adherence of *C. albicans* GDH 2346 to acrylic stored in a moist chamber (●) or in filter-sterilised mixed pooled saliva (○). Bars represent + and - 1 SEM.

showed that adherence of *C. albicans* to serum-coated acrylic strips was enhanced, while that to saliva-coated acrylic was inhibited when compared with serum- or saliva-free control strips. The results reported here confirm these results with a range of *Candida* species. Furthermore, when the *Candida* species were treated with saliva by suspending the organisms in saliva instead of PBS, adherence to acrylic was significantly lower than to untreated acrylic (data not shown), as previously shown with *C. albicans* (McCourtie and Douglas, 1981). The attachment of certain oral bacteria to hydroxyapatite is similarly inhibited by saliva (Clark and Gibbons, 1977; Gibbons and van Houte, 1980). Our results also support the in-situ observations of Olsen and Haanaes (1977) that yeast colonisation of acrylic plates worn by monkeys with a reduced salivary flow was increased. Furthermore, chronic oral candidal infections are commonly seen in patients with Sjögrens syndrome, whose salivary flow is absent or minimal (MacFarlane and Mason, 1974).

Roberts and Addy (1981) and Hennessey (1973) found that serum markedly reduced the bactericidal action of chlorhexidine. Although in our study serum and saliva considerably reduced the fungi-

cidal activity of chlorhexidine for all isolates of *Candida* tested, they had little effect on the ability of chlorhexidine to inhibit adherence of *Candida* species to acrylic when compared with chlorhexidine-mediated inhibition of adherence to untreated denture acrylic (McCourtie *et al.*, 1985).

It is thought that chlorhexidine is effective as an oral disinfectant because it is retained on oral surfaces and then slowly released into the oral cavity (Bonesvoll *et al.*, 1974a). After a 1-min rinse with 10 ml of chlorhexidine 0.2%, Bonesvoll *et al.* (1974a) found a sharp fall in the concentrations of chlorhexidine in saliva during the first few hours followed by slow release with bactericidal activity still present after 24 h. Oral retention was approximately proportional to the concentration of chlorhexidine used in the range tested (0.05–0.4%; Bonesvoll *et al.*, 1974b). With the higher concentrations of chlorhexidine used in this study (2%) inhibition of adherence of *C. albicans* to denture acrylic persisted for 19 days after treatment of the acrylic. However it is likely that *in vivo* the constant washing action of saliva would reduce this period of activity. It is interesting to note in this context that the application of a chlorhexidine acetate-impregnated polymer film to the surface of a removable partial denture significantly reduced plaque formation *in vivo* for up to 12 days while in-vitro growth of *Streptococcus mutans* was inhibited for as long as 45 days (Hirschfeld *et al.*, 1984). Moreover Schaeken *et al.* (1984) found that teeth treated for 5 min with chlorhexidine gluconate 5% in carboxymethylcellulose gel (the gel reduces the release rate of chlorhexidine) significantly reduced the numbers of *Str. mutans* for 21 days, compared with counts at untreated sites.

The results of this investigation coupled with our previous work (McCourtie *et al.*, 1985) suggest strongly that soaking acrylic dentures with chlorhexidine gluconate 2% for 15–30 min should be an effective measure in the treatment of CAC. The regular use of this would be useful in preventing a recurrence of CAC in patients susceptible to this condition.

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