Evaluation of the sanitization effectiveness of a denture-cleaning product on dentures contaminated with known microbial flora. An in vitro study

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Objectives: To see if dentures contaminated with *Staphylococcus aureus*, *Pseudomas aeruginosa*, *Bacillus cereus*, *Candida albicans*, and herpes simplex virus 1 could be effectively decontaminated by using Medical Tabs for Dentures. **Method and materials:** Ten methylmethacrylate dentures with processed soft liners (soft-liner dentures) and 10 methylmethacrylate dentures without processed soft liners (hard dentures) were aseptically fragmented and individually incubated with a target microorganism. Test denture fragments were immersed in Medical for 5 minutes, vortexed for 5 minutes, and serially diluted onto media. The control denture fragments were similarly treated in sterile water. For virus contamination, denture fragments were contaminated with 1.2×10^9 tissue culture infective dose $(TCID)_{50}$/mL. They were treated with either Medical for 5 minutes (test fragments) or water (controls) for 5 minutes. Serial dilutions were performed and viral $(TCID)_{50}$/mL titers were calculated using the Reed-Muench method. **Results:** Medical treatments effectively eliminated *C albicans*, *S aureus*, and *P aeruginosa* from soft-liner dentures. Treatment of hard dentures eradicated *C albicans* and reduced the numbers of *S aureus* and *P aeruginosa* to < 10. *B cereus* showed a reduction of 10 microorganisms in hard dentures while the soft-liner dentures did not show an appreciable reduction. Viral analyses found that both types of dentures retained large amounts of virus when washed with water, but no virus was recovered from any of the 40 samples treated with Medical. **Conclusion:** A single use of Medical Tabs for Dentures is effective in eliminating certain species of microorganisms, including selected viruses, in vitro. *(Quintessence Int 2004;35:194–199)*

Key words: denture-borne infection, denture cleaner, denture decontamination, denture stomatitis, disease transmission, iatrogenic infection, nosocomial infection, opportunistic infection

CLINICAL RELEVANCE: Control of the opportunistic and pathogenic microorganisms used in this study is essential to maintaining both good oral and general health of denture wearers.

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In the early 1980s, Glass and coworkers began studying the microorganisms that were associated with patients presenting with denture stomatitis, an infectious/inflammatory disease expressed on oral mucosa either directly under or in contact with the denture materials (eg, vestibules, buccal mucosae). They found that once the pathogenic microorganisms had been identified, the patients could be successfully treated with appropriate antimicrobial drugs. However, when the patients were taken off the drugs, the symptoms and numbers of microorganisms returned to their previous levels. These investigators expanded their clinical experience to examine in vitro denture contamination and found that *Candida albicans* contaminated not only the surfaces of the denture base material but the porosities as well. They postulated that denture contamination was primarily responsible for the recurrence of the mucosal infections.[1–3] They tried a variety of procedures to interrupt this cycle of reinfection by decontaminating the denture base material, especially

the porosities. The techniques utilized included commercially available denture-cleaning products, boiling water, and microwaving the dentures.[4–7]

Subsequent studies[8,9] have delineated the spectrum of microorganisms found in dentures that had been worn for time periods ranging from 12 days to over 40 years. Surprisingly, the most frequently isolated yeast in these studies was *C glabrata*, not *C albicans*. *C glabrata*, unfortunately, has a well-documented resistance to fluconazole (Diflucan, Pfizer). Equally important, a number of other opportunistic and pathogenic microorganisms were also present, including large numbers of gram-negative bacteria that can lead to endotoxic sepsis and systemic diseases.[8,9]

During the course of this latter study[9] some fragments of these dentures were not evaluated and were subsequently dried and then frozen (–80°C) for 1.5 years. When these samples were thawed and inoculated into appropriate microbial media, the spectrum of microorganisms found was similar to that found in the original study and the absolute numbers of microorganisms were only slightly reduced.[10] These findings indicated that the microbial populations found in the dentures are not significantly affected by harsh conditions such as desiccation and freezing. Pasteurization of these same denture fragments resulted in the subsequent growth of previously undetected spore-forming bacteria such as *Bacillus cereus* and *Clostridium perfringens*. Both of these species produce a plethora of harmful toxins, including lipases, hemolysins, and enterotoxins. Further experiments[11] by these investigators found that the effectiveness of certain denture cleaners could be significantly enhanced by the addition of 2 minutes of microwaving. Unfortunately, application of this technique is limited to nonmetallic dentures and dentures without soft liners.

Even though viruses were not identified in previously worn dentures, experiments with toothbrushes found that viruses can be retained on toothbrushes in substantial numbers for more than 7 days.[12] If viruses can contaminate toothbrushes, they might also contaminate dentures and be a factor in the pathogenesis of both oral and systemic diseases. Therefore, a comprehensive examination of the effectiveness of any denture-sanitizing agent should also address the decontamination of dentures contaminated with viruses. Due to the uncertainty of denture viral populations in vivo, experimental conditions were standardized by using denture fragments that had been contaminated in vitro with known concentrations of viral particles. A well-documented strain of a typical envelope virus commonly found in the human oral cavity, herpes simplex virus 1 (HSV-1, KOS strain), was chosen as a test agent.[13]

In Europe, Medical Tabs for Dentures (Medical , Bonyf AG) is a commercially available denture-sanitizing product. The efficacy of this product was examined by addressing the following research question:

Can dentures contaminated with the following microorganisms:

•*Staphylococcus aureus* (gram-positive cocci)
•*Pseudomas aeruginosa* (gram-negative rod)
•*Bacillus cereus* (gram-positive spore former)
•*Candida albicans* (yeast)
•Herpes simplex virus 1 (HSV-1, KOS strain)

be effectively decontaminated by using Medical?

METHOD AND MATERIALS

Processing dentures

Bonyf AG provided the study with a total of 10 methylmethacrylate dentures without soft liners (hard dentures) and 10 methylmethacrylate dentures containing processed soft liners (soft-liner dentures). The initial sterility of these unused dentures was established by inoculating blood agar plates (BAP) with denture surfaces and depths (cut dentures exposing porosities) and observing a lack of microbial growth. All experiments were performed with denture fragments obtained by aseptically sectioning the respective denture types (hard and soft-liner dentures) into multiple fragments of equivalent size. Separate experiments were performed on hard and soft-liner dentures for each of the species of microorganisms used in the study.

Experiments began by placing 20 to 22 grams of denture fragments into 100 mL of brain heart infusion (BHI) broth. The broth-denture mixture was then inoculated with an overnight culture of the test microorganisms, to achieve an initial concentration of approximately 10^6 microorganisms/mL. This mixture of denture fragments and microorganisms was incubated for 72 hours at 37°C with vigorous aeration. At the midpoint (36 hours), the supernatant was drained and replaced with fresh BHI broth to ensure that at least two complete microbial growth cycles occurred for each set of denture fragments. At the end of 72 hours, the supernatant was removed, the denture fragments were dried in sterile petri plates to a constant weight, and the fragments were subdivided into two equal groups.

The test groups of denture fragments (10 g each) were placed into 10 mL of test solution containing Medical used in accordance with the manufacturer's instructions (one tablet/160 mL 37°C sterile water). After 5 minutes of incubation in Medical solution, the test group was vortexed for 5 minutes. The control groups of denture fragments (10 g each) were placed

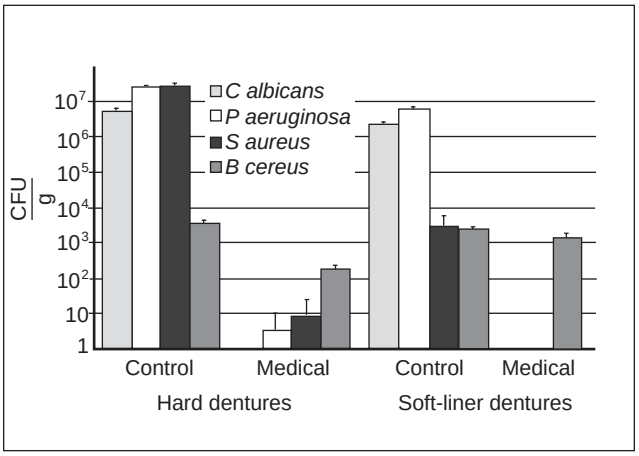

Fig 1 Comparative investigation of denture fragments contaminated by selected microorganisms and sanitized by Medical. The columns represent the average amount of pathogen eluted after treatment, while the error bars indicated one standard deviation. No detectable *C albicans* particles were found in the Medical-treated hard denture fragments. No *C albicans* particles were found in Medical-treated hard dentures, and no *C albicans*, *P aeruginosa*, or *S aureus* particles were found in the Medical-treated dentures with soft liners. CFU = colony-forming unit.

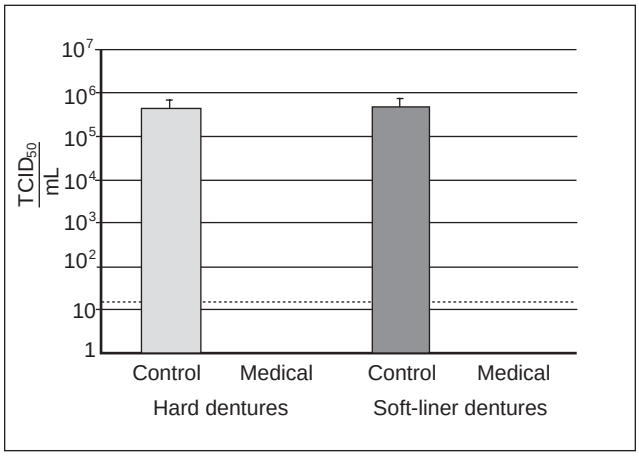

Fig 2 Comparative investigation of denture fragments contaminated with HSV-1 and sanitized with Medical. The columns represent the average amount of virus eluted after treatment, while the error bars indicate one standard deviation. The minimum quantity of virus, detectable in the assay system, is shown by the dotted line. No detectable virus particles were found in the Medical-treated denture fragments.

into 10 mL of sterile 37°C water for 5 minutes and vortexed for 5 minutes.

The number of microorganisms surviving the respective treatments was determined by standard procedures whereby test and control solutions were subjected to tenfold serial-dilutions in sterile water. Five separate one-mL samples were taken from each 10-mL test and control solution. Each aliquot was diluted from 1 to 10^{-7} and plated onto either trypticase soy agar plates with 5% sheep blood (*B cereus*, *P aeruginosa*, *S aureus*) or Sabouraud dextrose agar (*C albicans*). Duplicate samples were taken at each dilution resulting in a total of 10 values for each dilution for each species of microorganisms. All plates were incubated overnight at 37°C at an increased CO_2 concentration. After 24 hours of incubation, the number of colony-forming units (CFUs) were counted, recorded, and standardized according to weight (CFU/g of denture material). The results were subjected to appropriate statistical analyses.

For the in vitro HSV-1 viral analyses, 0.5 g of methylmethacrylate denture fragments (hard or soft liner) were placed into 80 (2×40) sterile, capped 16×100 mm polycarbonate tubes, sterilized with 70% ethanol, and allowed to dry overnight in a laminar flow hood. Each tube was contaminated with 1.2×10^9 tissue culture infective dose $(TCID)_{50}$/mL (HSV-1), at ambient temperature for 4 hours. One tablet of Medical was dissolved in 160 mL of 37°C water according to manufacturer's instructions. In the test groups, the viral inocula were aspirated from each tube and 2 mL of Medical solution was added. The tubes were vortexed and allowed to stand for 5 minutes at room temperature. The Medical solutions were aspirated and replaced with 2 mL of cell growth media (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/mL, 100 µg streptomycin/mL) and incubated for 3 hours at room temperature. The DME media was removed and quantified by viral titration. The control groups were treated in an identical manner except that 2 mL of sterile water was used in place of the Medical treatment.

Both media were diluted tenfold serially to 10^{-5}. Fifty microliters of undiluted media and 50 µL of each dilution were added to eight wells of a 96-well tray of Vero cells. The trays were maintained at 37°C in a 5% CO_2 incubator for 72 hours and scored for viral growth. Viral $(TCID)_{50}$/mL titers were calculated using the Reed-Muench method.[14]

Statistical analysis

Arithmetic means and standard deviations were calculated by standard procedures for each treatment and control, and the results are summarized in Figs 1 and 2.

Electron microscopy

Representative sections of selected denture fragments were examined by scanning electron microscopy (SEM). Samples were mounted on aluminum stubs and coated with a 60-nm mixture of gold/palladium using a Techniques Hummer V sputter coater (Polaron). The coated samples were scanned using a JEOL JXA-35 SEM. Photography was accomplished using a Polaroid 55 camera setup.

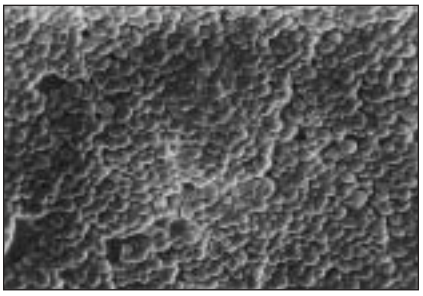

Fig 3a SEM photomicrograph demonstrating large aggregates of *C albicans* on the surface of an untreated soft-liner denture tooth (original magnification ×1,000).

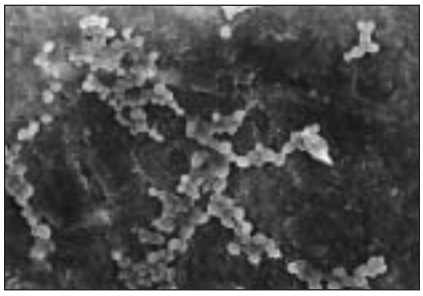

Fig 3b SEM photomicrograph demonstrating aggregates of *C albicans* on the surface of a water-treated hard denture. Note that some of the microorganisms have been washed away (original magnification ×1,000).

Fig 3c SEM of *C albicans* on the surface of a Medical-treated hard denture. Note the complete absence of microorganisms. Because no microorganisms were detected in the supernatant, it appears that Medical-treatment not only washed the microorganisms off the denture, but also directly killed them (original magnification ×1,000).

RESULTS

One treatment of Medical effectively eliminated *C albicans*, *S aureus*, and *P aeruginosa* from in vitro contaminated soft-liner dentures (Fig 1). One treatment of Medical applied to in vitro–contaminated hard dentures eradicated *C albicans* and reduced the numbers of CFUs in *S aureus* and *P aeruginosa* preparations from $> 10^7$ to $< 10^1$. One Medical treatment of *B cereus*–contaminated denture fragments resulted in a 10^1 reduction in microorganisms in hard dentures, while the soft-liner dentures did not show an appreciable reduction. It was noted that all four microbial species consistently grew better with hard denture fragments than with their soft-liner denture counterparts. These differences were most prominent in experiments involving *S aureus* (Fig 1). This correlation suggests that newly manufactured, pristine soft liners have substances with inherent antimicrobial properties. This was shown to be the case in preliminary experiments of this study in which pristine soft-liner material was removed from denture base material and found to be mildly inhibitory when added to growing microbial cultures (unpublished data).

The presence of viable microorganisms on the surfaces and fresh-cut edges (porosities) of hard and soft-liner denture fragments was confirmed by touching representative samples of each denture type to appropriate media after every treatment. Following treatment with Medical, there was no observable growth of either *C albicans* or *P aeruginosa*, minimal growth of *S aureus*, but abundant growth of *B cereus*. This protocol, which detects viable cells adhering to either the surface or porosities of denture fragments following treatment, was in general agreement with the comparable plate counts that enumerated living cells in the supernatant. The control (water-treated) fragments produced abundant growth on both the surfaces and fresh-cut edges (porosities) of all samples.

When the denture-touch observations and plate count results were compared with the concomitant SEM studies of the water-treated fragments, substantial numbers of microorganisms were observed on the surfaces and the interstices of these untreated dentures (Figs 3a and 3b). SEM photomicrographs of denture fragments after Medical treatment showed no microbial cells present (Fig 3c). The absence of living cells in either the supernatant or denture matrix indicates that Medical treatment is not only capable of physically removing susceptible microorganisms from the surfaces and depths of dentures, but can kill them as well.

Finally, *B cereus* appeared to be the least affected by the one-time use of Medical (Fig 1). It should be noted that the level of contamination by *B cereus* was inexplicably lower than that achieved with the other three species. Treatment of hard dentures resulted in only a 10^1-reduction in microorganisms, while the dentures with soft liners did not have a demonstrable reduction of microorganisms. One possible explanation for this apparently reduced level of contamination and decontamination (relative to the other species) may be the ability of *B cereus* to produce endospores. It is likely that many of the vegetative forms of *B cereus* were converted into endospores during the prolonged incubation period (72 hours) in these experiments. The resultant endospores would be highly resistant to a single exposure of a chemical agent (Figs 4a and 4b).

For the viral analyses, hard and soft-liner dentures were successfully contaminated with 1.2×10^9 $TCID_{50}$ of HSV-1. No viruses were recovered from any of the 40 samples following 5 minutes of treatment with

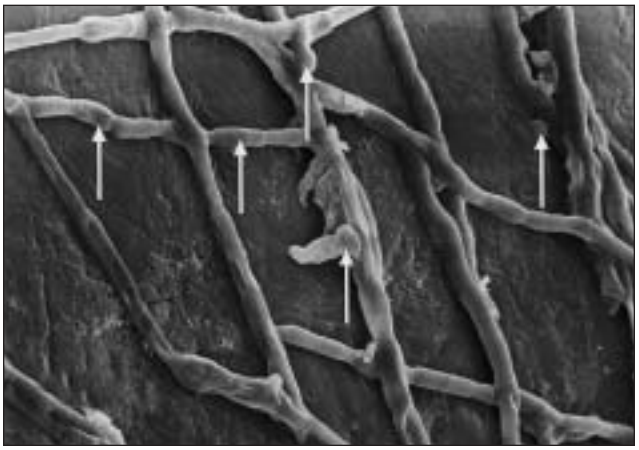

Fig 4a SEM photomicrograph demonstrating large networks of *B. cereus* on the surface of a water-treated soft-liner denture tooth. The broadened areas of the bacilli probably represent the presence of endospores *(arrows)* (original magnification ×1,000).

Fig 4b SEM photomicrograph demonstrating aggregates of *B. cereus* retained on the surface of a Medical-treated soft-liner denture. Note that while some of the microorganisms have been washed away, clearly vegetative forms of the microorganism still remain *(arrows)* (original magnification ×1,000).

Medical (Fig 2). The minimum detectable level of virus under these experimental conditions is 20 infectious particles. This means that a single treatment with Medical resulted in a > 99.99% reduction in the number of infectious HSV-1 virus particles in contaminated hard and soft-liner dentures. Both types of dentures retained large amounts of virus ($> 10^5$) when washed with water.

DISCUSSION

The objective of this study was to determine the efficacy of a commercial product (Medical) to decontaminate dentures that had been colonized by species of pathogenic and opportunistic microorganisms. This objective was accomplished by individually contaminating newly manufactured dentures with pure cultures of target microorganisms and then evaluating the efficacy of Medical to either remove and/or kill the offending microbe.

Preliminary studies determined that in vitro contamination of intact newly manufactured dentures from Europe resulted in such low levels of contamination that it would be difficult to measure the differential between treated and untreated dentures. These low levels of in vitro contamination are quite likely reflections of the essential roles that mastication forces and oral environment play in denture contamination in vivo, and/or may have been due to the European-made dentures not having the "personalizing fibers" of American-made dentures. The obstacle of low levels of denture contamination was overcome by aseptically macerating intact dentures into fragments, which enhanced contamination by increasing the surface area exposed to the target microorganisms.

The results of this study indicate that Medical is very effective against *C albicans* (yeast), *P aeruginosa* (gram-negative rod), and *S aureus* (gram-positive cocci). These species are well-known pathogens responsible for significant oral and systemic infectious diseases in man. Medical was much less effective against *B cereus* (gram-positive spore-forming rod), which is associated with gastroenteritis. The molecular basis of this resistance was not directly addressed in this study. One possible explanation for the survival of *B cereus* would be that the vulnerable vegetative cells are routinely converted to highly refractive endospores during the prolonged stationary phase of growth inherent in these experiments. In a nutritionally rich environment such as the oral cavity, these protective endospores would be expected to germinate and return to the vegetative form. If this hypothesis is true, daily denture treatments with Medical could eventually eradicate a population of spore-forming bacteria by eliminating them while they are in the susceptible vegetative state.

When undiluted media from the Medical-treated dentures were placed onto Vero cells, there was strong inhibition of cellular growth, but no cellular death. This growth inhibition was not seen with a 10^{-1} or higher dilution of media. There was no growth inhibition when any of the media from the control-treated dentures were inoculated onto Vero cells. This suggests there is a toxic substance in the Medical that affects these robust Vero cells. However, toxicology studies on Medical revealed no evidence of irritation to human buccal mucosa that had been reconstituted in in vitro cell cultures of transformed keratinocytes (unpublished data).

The inhibition or toxicity of Medical for tissue cultures prohibited attempts to duplicate the in vitro contamination and treatment experiments using human cytomegalovirus (CMV) and a retrovirus. These viruses

are assayed using human fibroblast cells (MRC-5), a very sensitive, commonly used cell line. Media used to elute virus from the Medical-treated dentures killed the fibroblasts when either undiluted or at a 10^{-1} dilution, limiting the sensitivity of the assay to 2,000 infectious particles. To obtain statistically significant virus titers after treatment, the dentures would need to be contaminated with a minimum of 10^8 $TCID_{50}$ CMV or SFV-bab (a retrovirus). This viral dose could only be achieved by large-scale growth and concentration of these viruses that were outside of the scope of this study.

HSV-1 is an enveloped virus and seems to be particularly susceptible to the actions of Medical. It would be of interest to determine if non-enveloped oral viruses are susceptible to Medical treatment. One potential candidate would be mengovirus. This virus is a non-enveloped member of the Picornaviridae, a large virus family that includes significant pathogens such as poliovirus, hepatitis A, and rhinovirus. Mengovirus has the added advantage of growing to very high titers and can be readily assayed on Vero cells.

CONCLUSIONS

1. Methylmethacrylate dentures (hard dentures) and methylmethacrylate dentures with processed soft liner (soft-liner dentures) can be fragmented and reproducibly contaminated with known quantities of bacteria, yeasts, and viruses in a model system to determine the ability of Medical to decontaminate denture fragments in vitro.
2. Medical treatments eliminated *C albicans*, *S aureus*, and *P aeruginosa* from in vitro contaminated soft-liner denture fragments.
3. Medical treatments of in vitro contaminated hard denture fragments eliminated *C albicans* and substantially reduced populations of *S aureus* and *P aeruginosa*.
4. The least responsive microorganism to Medical treatment was *B cereus*. However, this finding may be skewed by the formation of protective endospores.
5. Medical treatments were consistently effective in killing the HSV-1 target virus.

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