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Title: Effect of surface treatments of titanium on amphotericin B-treated *Candida albicans* persister cells.

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Short title: *C. albicans persister cells on titanium* surface

Keywords:

Titanium, *Candida albicans*, biofilm, surface treatments, antifungal resistance

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Summary

Background: Although persister cells in *C. albicans* biofilm may contribute to its increased resistance to antifungal drugs, little information is available on the formation of *Candida* persister cells on titanium surfaces. Objective: The effect of different surface treatments of Ti on persister cells was determined in the current study. Methods: Titanium discs were surface-treated by three different methods (Group A - polishing, Group B - sandblasting followed by acid-etching, and Group C - sandblasting alone). Persister cells of two *C. albicans* strains, namely ATCC 90028 and HK30Aa, in biofilm and planktonic states, was measured as the percentage of colony forming units remaining after 24 h incubation with various concentrations of amphotericin B. Results: No persister cells were detected in the planktonic cultures. However, 1.5%, 0.1% and 2.4% *C. albicans* ATCC 90028 persister cells were detected at a AmB concentration of 64 µg/ml in groups A, B and C, respectively; and 0.3%, 0.2% and 0.6% for groups A, B and C, respectively, for HK30Aa. Conclusions: Group C of *C. albicans* ATCC 90028 appeared to provide a surface relatively unfavorable for persister cells development ($p < 0.01$). Whether these results may have implications on the clinical performance of titanium implants warrants further investigation.

Introduction

Microorganisms exist predominantly as biofilms rather than as planktonic or free-floating cells in most natural environments [1]. The insertion of implants and prosthetic devices such as catheters, prosthetic heart valves and prosthetic joints into patients provides pathogenic microorganisms, such as *Candida*, with a substratum on which they can form biofilms. An important property of biofilms is that their constituent microorganisms may become more resistant to antimicrobials than their planktonic counterparts [2], which poses a serious clinical problem. In dentistry, ~~titanium (Ti) is commonly used for maxillofacial reconstructions and in dental implants to replace missing teeth and affix dental prostheses, because of its strength, low density and corrosion resistance [3].~~ a~~A~~lthough dental Ti implants generally have a high long-term clinical success rate, clinical studies have shown an association between specific microorganisms and implant failure [~~4~~3-~~6~~5]. This finding may be related to the surface finishing of the Ti before use. Having a rough surface has been shown to promote osseointegration [~~7~~6]; yet, bacterial adhesion and colonization can be promoted once rough Ti surfaces become exposed to the oral cavity [~~8~~7]. ~~Increased microbial adherence and colonization have indeed been considered to be key factors in the pathogenesis of biomaterial-based infection [9].~~

The characteristics of implant surfaces thus seem important in both osseointegration and infection. ~~However, it may not be possible to minimize the potential for the latter, because~~

~~reducing the surface roughness of implants below a certain threshold value (Ra = 0.2 μm) has no major effect on the composition of supra- and sub-gingival microorganisms [10].~~ A ~~further~~ consequence of surface roughness relates to antimicrobial susceptibility and the development of resistance among biofilm microorganisms. Different sandblasted Ti surfaces can affect antifungal resistance of *Candida* biofilms [~~11~~8]. Such phenomenon would be relevant to the use and care of Ti implants in dentistry.

~~The mechanisms by which biofilms become resistant to antifungal agents are still not fully understood. Widely accepted mechanisms are altered physiological state of fungal cells, barrier function of the extracellular matrix (ECM), over-expression of membrane-localized drug efflux pumps and variations in fungal membrane sterol composition [12-14].~~

Recently, researchers have found that the existence of persister cells in *C. albicans* biofilms may contribute to increased resistance to antifungal drugs [~~15~~9]. However, little information is available on the formation of *Candida* persister cells on Ti with different surface treatments. The current study therefore aimed to investigate whether *C. albicans* persister cell can be detected on Ti surfaces. The effect of different surface treatments of Ti on the number of persister cells was also determined.

Materials and Methods

Candida isolates

Two *C. albicans* strains, ATCC 90028 and HK30Aa, were used in this study. HK30Aa was a clinical strain isolated from a patient with asymptomatic HIV infection.

Preparation of Ti discs

Commercially pure Ti rods (Arkhe, Fukui, Japan) were cut into discs of 12-mm diameter and 1-mm thickness with a wire-cut electrical discharge machine (Agie Charmilles, Nidau, Switzerland). The discs were divided into the following three surface-treatment groups:

Group A: polished surface

Discs were polished with silicon carbide waterproof abrasive paper (roughness grade, 1200 CW). The discs were then finely polished with an Al_2O_3 grinding wheel.

Group B: Sandblasted and acid-etched surface

Discs were first sandblasted with 99.6% Al_2O_3 (Korox; Bego, Bremen, Germany) with a mean grit size of 250 µm. Sandblasting was done with a sandblasting machine (Dentastrah Combi; Krupp Medizinteehulk, Germany) at a pressure of 5 bar for 30 s at a distance of 1.5 cm. The discs were then etched with a mixture of 2% (v/v) hydrofluoric acid and 10% (v/v) nitric acid for 3 min [~~16~~10].

Group C: Sandblasted surface

Discs were sandblasted with 99.6% Al_2O_3 with a mean grit size of 250 µm, as described in group B.

Ti discs after surface treatments were rinsed with distilled water, then 70% (v/v) ethanol and 99% (v/v) ethanol for 15 s. The Ti discs were then immersed in 99% ethanol in a sonicating bath for 15 min. After the discs were cleaned, they were packed into autoclave bags and autoclaved at 121°C for 15 min.

Profilometric analysis of Ti discs

Two discs from each group were randomly selected for profilometric analysis in a stylus profiler (Surtronic 3+; Taylor Hobson Precision, Leicester, UK) to determine their surface roughness (Ra) [~~11~~8]. Three measurements were taken on each disc and the mean Ra value was calculated.

Scanning electron microscopy (SEM) study of Ti disc surfaces

One randomly selected Ti disc from each group was viewed with SEM (XL-30CP; Philips, Cambridge, UK). The Ti discs were coated with gold and imaged in a high-vacuum mode at

10 kV. Scanning electron micrographs of the disc surfaces were taken at 100× magnification.

Adhesion assay

Candida albicans ATCC 90028 and HK30Aa were subcultured and grown on Sabouraud dextrose agar plate at 37^{o}C for 24 h. Cells were then grown in yeast nitrogen base (YNB, Difco, Maryland, USA) with 50 mM glucose w/v at 37°C in an orbital shaker at 60 rpm. After 24 h, the cells were washed twice in PBS and resuspended to an optical density of 0.385 at 520 nm.

The Ti discs from groups A, B and C were placed in a 12-well polystyrene plate (Iwaki-Asahi Techno Glass, Tokyo, Japan) and 500 μl of prepared yeast suspensions were added on the disc surface. Candidal cells were allowed to adhere on the disc surface for 90 min at 37°C in an orbital shaker incubator at 75 rev/min. Each Ti disc was picked up using sterile artery forceps and gently washed in 350 ml of PBS in a sterile container by back and forth movement for 2 s to remove non-adherent cells. The adhered cells were harvested from the Ti discs by curettage and vortexing, washed twice in PBS and resuspended in 100 μl of PBS. Viable counts were then determined by serial dilution and plating on Sabouraud dextrose agar plates.

Biofilm formation

The adhesion phase of candidal biofilm formation was performed as described in the adhesion assays. The Ti discs were then washed once as described and placed in a new pre-sterilized, 12-well polystyrene plate with 2 ml of YNB supplemented with 50 mM glucose. The discs were incubated at 37^{o}C and 75 rpm for 48 h in an orbital shake incubator. Biofilm cells were then harvested from the Ti discs by curettage and vortexing, washed twice in PBS and resuspended in 100 µl of PBS. Viable counts were then determined by serial dilution and plating on Sabouraud dextrose agar plates.

Amphotericin B treatment of C. albicans biofilms

Candida biofilms on Ti discs were placed into a 12-well polystyrene plate, with wells containing 2 ml of YNB glucose medium with amphotericin B (AmB) at different concentrations ranging from 0.5 to 64 µg/ml, buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (Sigma; St. Louis, Missouri, USA). The discs were incubated in the orbital shaker incubator at 75 rev/min for 24 h. *Candida albicans* biofilm with no AmB served as the control. Biofilm cells were then harvested from the Ti discs by curettage and vortexing, washed twice in PBS and resuspended in 100 µl of PBS. Viable counts were then determined by serial dilution and plating on Sabouraud dextrose agar plates. All assays were carried out on three different occasions in duplicate (A total of six samples

each).

Amphotericin treatment of planktonic C. albicans

ATCC 90028 and HK30Aa cell suspensions in YNB glucose medium as prepared in the adhesion assay were incubated with AmB at different concentrations ranging from 0.5 to 64 µg/ml. Suspensions without AmB served as negative controls. The samples were placed in an orbital shaker incubator at 37°C and 75 rev/min for 24 h. The cells were then washed twice in PBS and resuspended in 100 µl of PBS. Viable counts were then determined by serial dilution and plating on Sabouraud dextrose agar plates. All assays were carried out on three different occasions in duplicate (A total of six samples each).

Statistical analysis

Statistical analysis was conducted using one-way-ANOVA to compare the difference among the three groups. The Bonferroni multiple comparison test was also used. A *P* value of < 0.05 was considered to be statistically significant.

Results

Surface roughness of Ti discs

Of the three disc groups, those with polished surfaces (group A) had the lowest mean (SD) Ra values, of 0.85 ± 0.19 μm, whereas Al_2O_3-sandblasted discs (group C) had the highest values, of 3.80 ± 0.17 μm. The Ra of sandblasted and acid-etched discs (group B) was 2.33 ± 0.11 μm ($P < 0.001$).

SEM of Ti discs after different surface treatments

Group A discs had a relatively smooth surface with well-defined unidirectional grooves, which are typically seen on machined implant surfaces (Fig. 1.i). Group B discs had a homogeneous undulating surface (Fig. 1.ii). Group C discs had a very rough surface with multiple small pits and grooves superimposed on larger cavities (Fig. 1.iii).

Adhesion assay

The mean (SD) counts of ATCC 90028 for groups A, B and C were $1.38 \times 10^5 \pm 72$, $1.94 \times 10^5 \pm 70$ and $1.41 \times 10^5 \pm 70$ cfu/100 μl, respectively. The mean counts of HK30Aa for groups A, B and C were $1.29 \times 10^5 \pm 64$, $1.47 \times 10^5 \pm 71$ and $1.73 \times 10^5 \pm 53$ cfu/100 μl, respectively. No statistically significant difference was noted between the three groups of either ATCC 90028 or HK30Aa.

Biofilm formation

The mean (SD) counts of ATCC 90028 for groups A, B and C were $3.42 \times 10^6 \pm 112$, $2.22 \times 10^6 \pm 56$ and $7.33 \times 10^6 \pm 241$ cfu/100 μl, respectively. The mean counts of HK30Aa for groups A, B and C were $4.70 \times 10^6 \pm 302$, $4.02 \times 10^6 \pm 578$ and $4.92 \times 10^6 \pm 150$ cfu/100 μl, respectively. A statistically significant difference was found between the biofilm cell counts in the three groups (group C > A > B) of ATCC 90028 ($P < 0.001$).

Amphotericin treatment of C. albicans biofilms

A small proportion of cells remained resistant to AmB concentration at 64 μg/ml. The percentage of persister cells found in the three groups for both ATCC 90028 and HK30Aa was found to be statistically significant different from each other, though the actual difference was small. (1.5%, 0.1% and 2.4%, respectively, for ATCC 90028; 0.3%, 0.2% and 0.6%, respectively, for HKAa) ($P < 0.01$). Figures 2 and 3 show the cfu counts at different concentrations of Am B for the two strains.

Amphotericin treatment of planktonic C. albicans

Both ATCC 90028 and HK30Aa showed a 100% reduction in cfu at an AmB concentration of 1 μg/ml. No persister cells could be detected at higher AmB concentrations.

Discussion

In this study, the antifungal resistance of *C. albicans* in planktonic form was found to be much lower than the biofilm form, regardless of the Ti surface and *C. albicans* strain (Both strains showed a 100% reduction in cfu at an AmB concentration of 1 μg/ml in its planktonic form while persister cells were detected at a concentration of 64 μg/ml in its biofilm form). This result agrees with those of other studies [~~18, 19~~11, 12]. ~~Chandra *et al.* showed a 32-fold increase in minimum inhibitory concentration in candidal biofilms on denture acrylic than their planktonic counterparts using a colorimetric XTT-metabolic assay [20]. The lower magnitude of antifungal resistance in our study could be because Ti surfaces were used instead of acrylic, and our candidal strains were different from those used by Chandra *et al.* The increased resistance of candidal biofilms does not seem to be attributable to the ECM, because the *C. albicans* strains we used (ATCC 90028 and HK30Aa) did not form any hyphae or observable ECM. Previous studies have suggested that even when ECM is present in biofilms, it does not hinder penetration of antifungal drugs [21] and the amount of ECM (controlled by growing cells with or without shaking) does not have a major role in biofilm resistance [22].~~

~~Although SEM clearly showed that the three types of Ti discs had distinct surface morphologies, *C. albicans* adhesion after 90 min was similar in the three groups. However, when we examined biofilm formation after 48 h, biofilms of ATCC 90028 in group C had a~~

~~higher cfu count than the other two groups.~~ The sandblasted Ti discs in group C had the roughest surfaces in this study, and their deep pits and cavities may have sheltered the yeast cells from the external environment better than those in the other discs. Indeed, the surfaces of group A and B discs may have been too smooth or homogeneous, and the features may have been too small or too large, to provide an effective shelter ~~Surface features that are much smaller or larger than microorganisms have little effect on cell retention~~ [~~23~~13]~~,~~ ~~perhaps because very small pits have limited shelter ability and very large pits behave too much like a flat surface~~. In addition, group B discs, which were sandblasted and acid-etched, had the lowest amount of biofilm growth after 48 h, significantly so for ATCC 90028 cells. Acid passivation makes surfaces more hydrophilic and reduces the extent of colonization by bacteria [~~24~~14], which preferably bind hydrophobic receptor sites. In our study, the hydrophilic nature of group B discs might have hindered candidal adhesion.

It is well known that adherence and biofilm formation are affected by pre-coating the substrate surface with saliva. Previous workers have demonstrated that coating the substrata with unstimulated whole saliva significantly inhibited adhesion [~~25-27~~15-17] but resulted in increased biofilm formation by *C. albicans* [~~28~~18].5. H. Nikawa, H. Nishimura, T. Hamada, H. Kumagai and L.P. Samaranayake, Effects of dietary sugars and, saliva and serum on *Candida* biofilm formation on acrylic surfaces. *Mycopathologia* **139** (1997), pp. 87–91. **Full Text** via CrossRef | View Record in Scopus | Cited By in Scopus (16) On the other hand, Jin

et al. found that the presence of a saliva coating (unstimulated whole saliva) did not significantly influence the degree of either *C. albicans* adhesion or biofilm growth [~~29~~19]. Due to these conflicting observations, in the current study the titanium discs were not pre-coated with saliva before the adhesion assay or biofilm growth.

Currently, the surface roughness of commercially available Ti implants at the region of the fixture is usually in the range of 0.5 to 10 µm [~~30~~20]. The roughness of the Ti discs in our study fell within this range, but surface morphology and roughness appeared to have no major effect on antifungal susceptibility of the candidal biofilms at first sight. Hence, if a region of a Ti implant becomes exposed during implant therapy, whether the surface had been polished, sandblasted or both sandblasted and acid-etched may have little effect on the biofilm susceptibility to antifungals. However, even at the highest concentration of AmB tested in this study (64 µg/ml), persister cells could be detected. These persister cells could be largely responsible for multidrug tolerance in candidal biofilm [~~15~~9].

Persister cells are present at a level of 0.1% to 1% in bacterial biofilms of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [~~31~~21]. ~~They can survive at concentrations of antibiotics that are well above the minimum inhibitory concentration and are phenotypic variants of wild type cells rather than mutants [32].~~ A recent study reported that *C. albicans* exhibits a biphasic killing pattern in response to AmB and chlorhexidine [~~15~~9]. The majority of cells were susceptible but a small subpopulation, about 1%, was

highly tolerant. Al-Dhaheri & Douglas [~~17~~22], on the other hand, demonstrated 0.01% of *C. albicans* was resistant to AmB at a concentration of 100 μg/ml.

~~Nevertheless, persister cells were absent in biofilms of *C. glabrata* or *C. tropicalis*.~~

Similar to the previous two studies, ~~no persister cells were detected in the planktonic cultures in the current study. Nevertheless,~~ persister cells were found in biofilms of both *C. albicans* ATCC 90028 and HK30Aa, though different titanium surface treatments had different levels of persister cells. Because mutants defective in biofilm formation can produce persister cells at a level similar to controls, substrate attachment rather than formation of a well-established biofilm seems to be the important factor in stimulating the formation of persister cells. Our study demonstrated ~~the existence of persister cells after treatment with AmB, with~~ the highest proportion of surviving cells on group C discs. The sheltering effect owing to the surface topography of sandblasted discs may encourage biofilm anchorage and the formation of persister cells. In contrast, the more hydrophilic surface of group B discs due to acid-etching may discourage biofilm anchorage and explain the lower number of persister cells. ~~Further experiments on the identification of genes responsible for the persister phenotype may help to explain how they develop.~~

~~Moreover, whether exposed Ti implants, especially those that have been sandblasted, may encourage the development, survival and propagation of persister yeast cells and impart an increase in antifungal resistance clinically warrants further investigation. Studies on finding~~

~~new methods to isolate, quantify and analyze these cells *in vivo* may shed light on this subject.~~

In conclusion, the current study demonstrated for the first time the presence of *C. albicans* persister cells on titanium surface. Although there were statistically significant differences in the percentage of persister cells between the three groups, whether there is any clinical significance warrants further investigations. Further studies such as finding new methods to isolate, quantify and analyze these cells *in vivo*, analyses of yeast cell attachment, and the formation and properties of persister cells would provide further insight into mechanisms of biofilm ~~susceptibility and~~ resistance to antifungals. Surface preparation may also need to aim for optimal implant osseointegration while minimizing antimicrobial resistance of biofilms.

Acknowledgements

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Figure legends

Figure 1 – Ti i) with polished surface, showing a relatively smooth surface with well-defined unidirectional grooves, which are typically seen on machined implant surfaces, ii) after sandblasting with 250-µm Al_2O_3 and acid-etching, had a homogeneous undulating surface, iii) after sandblasting with 250-µm Al_2O_3 had a very rough surface with multiple small pits and grooves superimposed on larger cavities, viewed under SEM (100× magnification).

Figure 2 – Log cfu counts of *C. albicans* ATCC 90028 exposed to different concentrations of AmB: (◇) - biofilm cells on polished, (□) - sandblasted and acid etched and (△) - sandblasted titanium surfaces; (×) – planktonic cells. Data are means ± standard error of three independent experiments performed in duplicate.

Figure 3 – Log cfu counts of *C. albicans* HK30Aa exposed to different concentrations of AmB: (◇) - biofilm cells on polished, (□) - sandblasted and acid etched, and (△) - sandblasted titanium surfaces; (×) – planktonic cells. Data are means ± standard error of three independent experiments performed in duplicate.

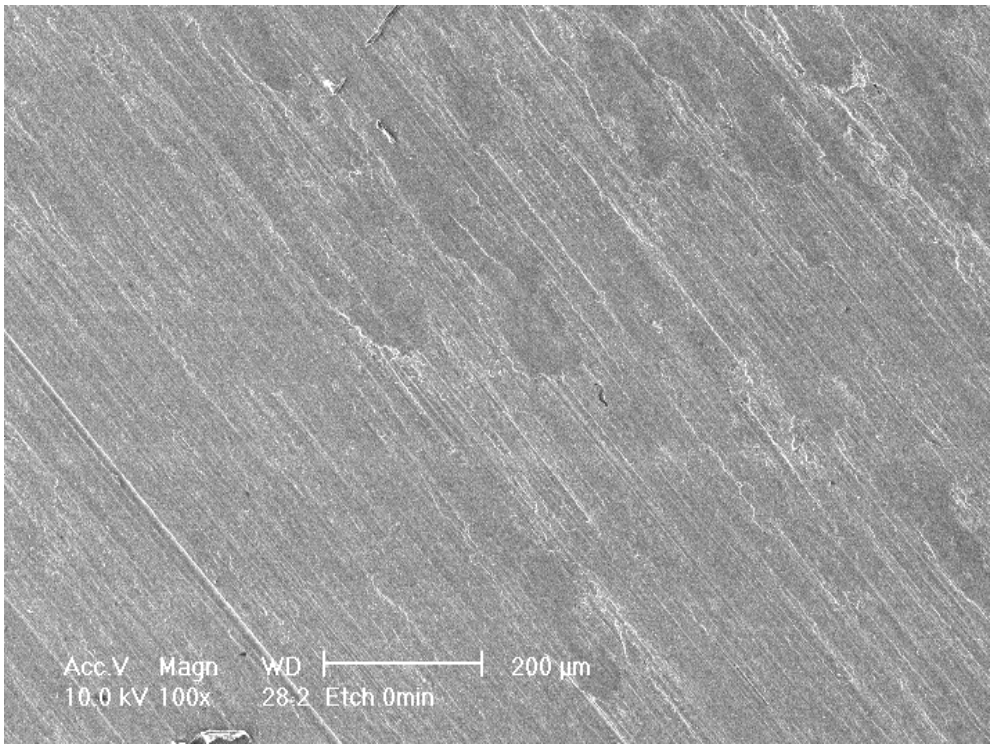


Figure 1.i

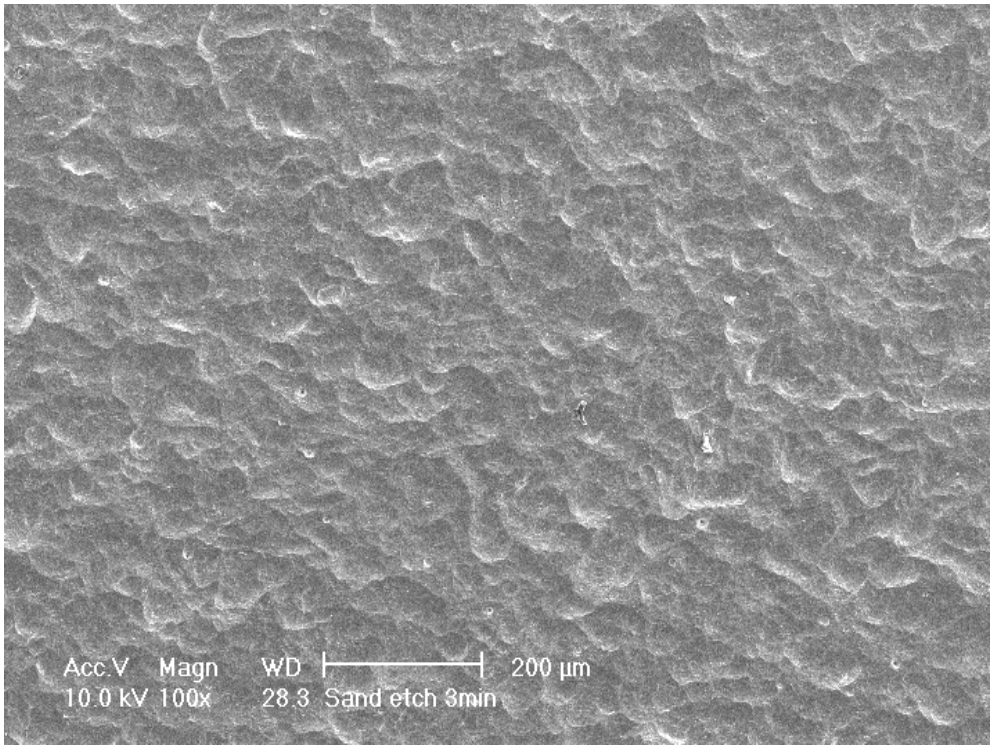


Figure 1.ii

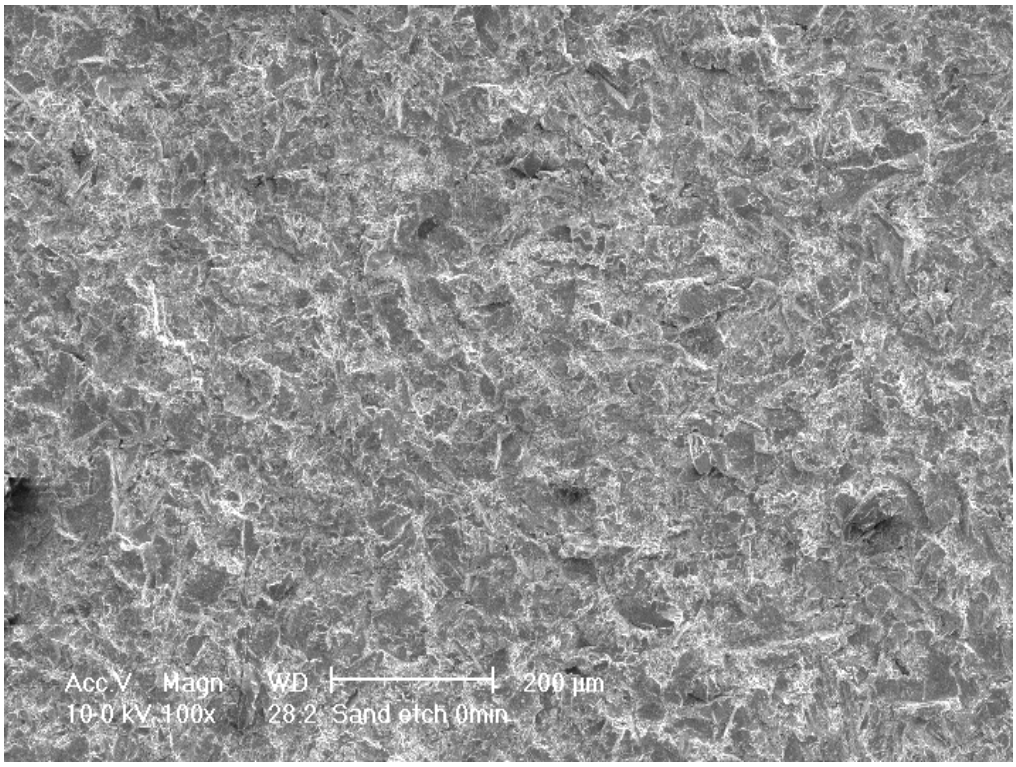
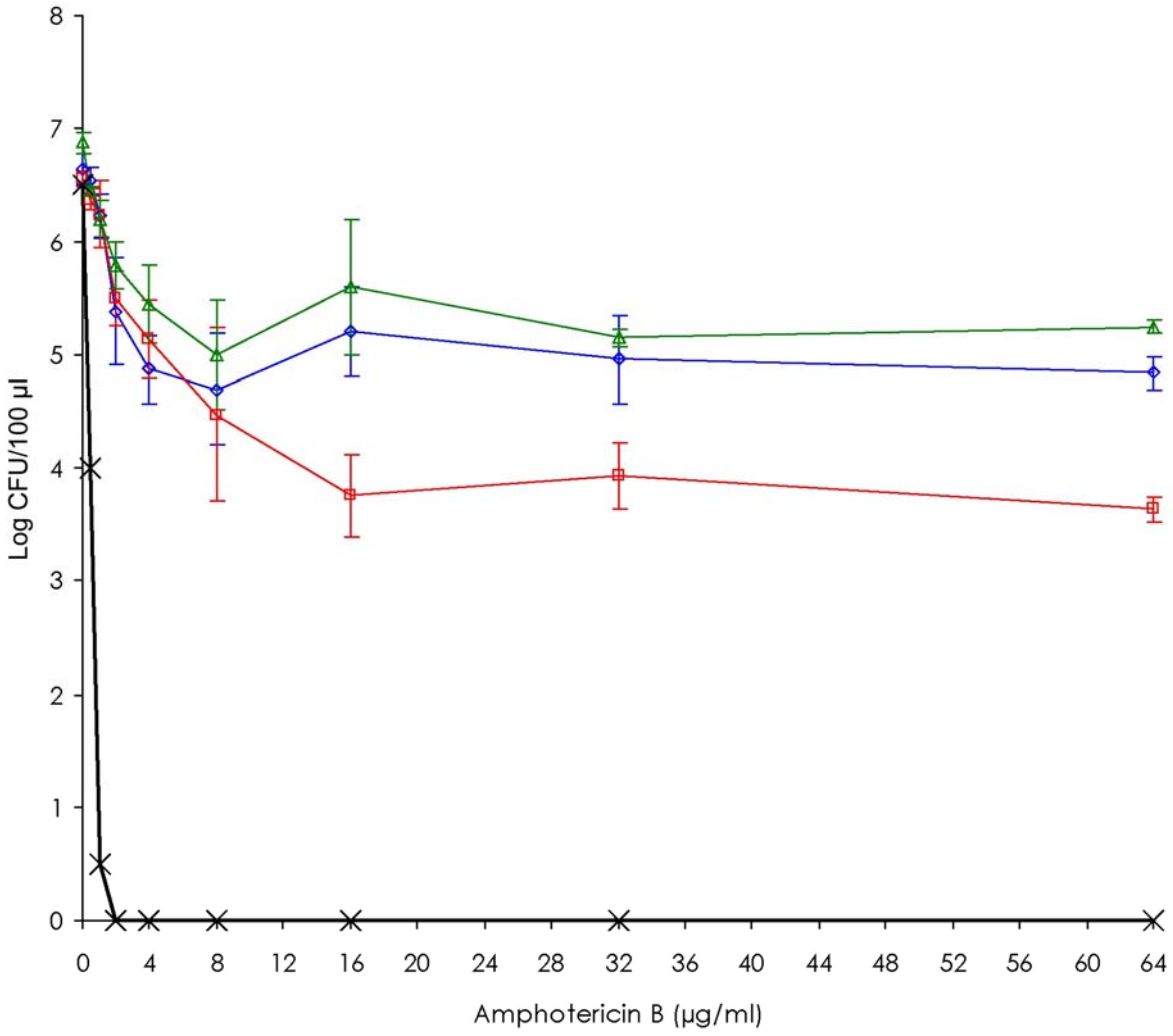


Figure 1.iii


Log CFU/100 µl
0
1
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7
8
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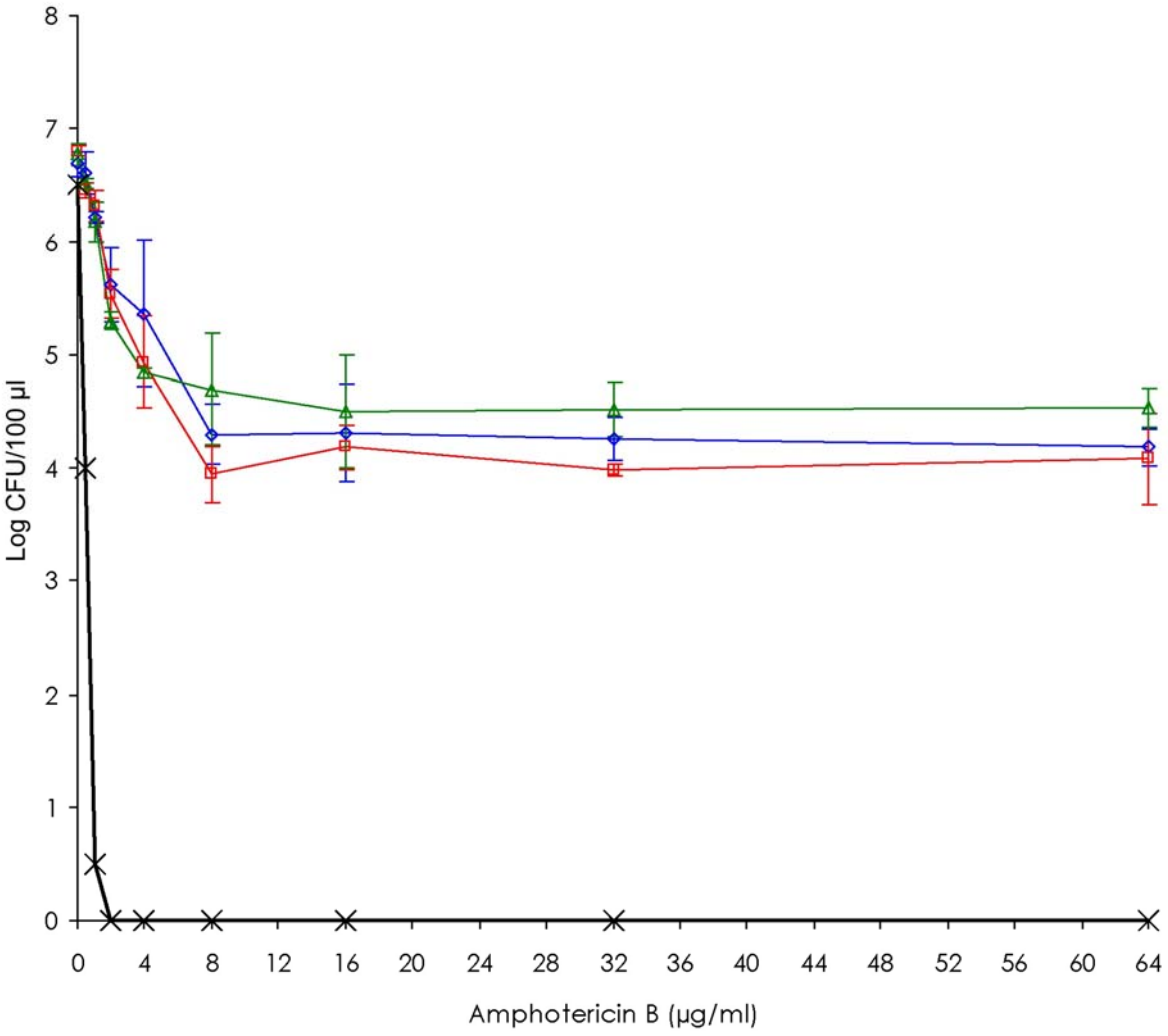
32
36
40
44
48
52
56
60
64
Amphotericin B (µg/ml)

Figure 2


Log CFU/100 µl
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40
44
48
52
56
60
64
Amphotericin B (µg/ml)

Figure 3