



Status of the effectiveness of contact lens disinfectants in Malaysia against keratitis-causing pathogens

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ABSTRACT

The aim of this study was (i) to assess the antimicrobial effects of contact lens disinfecting solutions marketed in Malaysia against common bacterial eye pathogens and as well as eye parasite, *Acanthamoeba castellanii*, and (ii) to determine whether targeting cyst wall would improve the efficacy of contact lens disinfectants. Using ISO 14729 Stand-Alone Test for disinfecting solutions, bactericidal and amoebicidal assays of six different contact lens solutions including Oxysept[®], AO SEPT PLUS, OPTI-FREE[®] pure moist[®], Renu[®] fresh[™], FreshKon[®] CLEAR and COMPLETE RevitaLens[™] were performed using Manufacturers Minimum recommended disinfection time (MRDT). The efficacy of contact lens solutions was determined against keratitis-causing microbes, namely: *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Acanthamoeba castellanii*. In addition, using chlorhexidine as an anti-amoebic compound and cellulase enzyme to disrupt cyst wall structure, we determined whether combination of both agents can enhance efficacy of marketed contact lens disinfectants against *A. castellanii* trophozoites and cysts, *in vitro*. The results revealed that all contact lens disinfectants tested showed potent bactericidal effects exhibiting 100% kill against all bacterial species tested. In contrast, none of the contact lens disinfectants had potent effects against *Acanthamoeba* cysts viability. When tested against trophozoites, two disinfectants, Oxysept Multipurpose and AO-sept Multipurpose showed partial amoebicidal effects. Using chlorhexidine as an anti-amoebic compound and cellulase enzyme to disrupt cyst wall structure, the findings revealed that combination of both agents in contact lens disinfectants abolished viability of *A. castellanii* cysts and trophozoites. Given the inefficacy of contact lens disinfectants tested in this study, these findings present a significant concern to public health. These findings revealed that targeting cyst wall by using cyst wall degrading molecules in contact lens disinfecting solutions will enhance their efficacy against this devastating eye infection.

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1. Introduction

Microbial keratitis is a serious vision-threatening infection. Clinical features include redness, pain, tearing, blur vision and inflammation but symptoms vary depending on the causative agent (Ansari et al., 2013; Stapleton et al., 2007; Stapleton and Carnt, 2012). It is caused by viral, bacterial, fungal, and protist pathogens. Contact lens use is one of the important risk factors in contracting microbial pathogens leading to microbial keratitis. This is of particular concerns for less developed countries where the use of contact lenses is on the rise, with limited awareness of associated risks. For example, recently we tested the effects of contact lens disinfectants marketed in Pakistan against microbial pathogens. The findings revealed that none of the commercially available contact lens disinfectants were effective against eye par-

asite, *Acanthamoeba castellanii* (Lakhundi et al., 2014). In part, this was attributed to the ability of *Acanthamoeba* parasite to transform into a phenotypically distinct cyst form under harsh conditions (Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007; Siddiqui and Khan, 2012). Cysts are double-walled, hardy structures with minimal metabolic activity making it resistant to harsh environmental conditions as well as biocides, resulting in prolonged treatment and often leads to infection recurrence Aksozek et al., 2002; Turner et al., 2004; Khan, 2006). Given a plethora of contact lens disinfectants available in the market, it is important to assess their antimicrobial properties. Among a number of pathogens responsible for microbial keratitis, here we tested the efficacy of contact lens disinfectants marketed in Malaysia including Oxysept[®], AO SEPT PLUS, OPTI-FREE[®] pure moist[®], Renu[®] fresh[™], FreshKon[®] CLEAR and COMPLETE RevitaLens[™] against common bacterial pathogens causing eye infections, including species of *Pseudomonas*, *Staphylococcus*, and *Streptococcus*, as well as leading eye parasite, *Acanthamoeba castellanii*. Moreover, as *Acanthamoeba* cyst walls are

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partly made of cellulose, we determined the inclusion of cellulase enzyme in contact lens disinfectants to degrade *Acanthamoeba* cyst walls in the improved efficacy of commercially available contact lens disinfectants.

2. Material and methods

All chemicals were purchased from Sigma Labs (Poole, Dorset, England) unless otherwise stated. The stock solution of chlorhexidine (CHX) was prepared in methanol and was stored at room temperature. We used cellulase enzyme isolated from *Trichoderma viride* (70 Units) and chlorhexidine dihydrochloride (50 μ M) in our experiments.

2.1. Contact lens disinfecting solutions

The type of contact lens disinfectants, the compositions and manufacturers' Minimum Recommended Disinfection Time (MRDT) are listed in Table 1. All disinfectants were examined before the expiry date recommended by the Manufacturer. Six different contact lens disinfectants included, Oxysept[®], AO SEPT PLUS, OPTI-FREE[®] pure moist[®], Renu[®] fresh[™], FreshKon[®] CLEAR and COMPLETE RevitaLens[™]. These solutions were commonly marketed in Malaysia and were bought from nearby local pharmacy.

2.2. Test organisms and growth conditions

Among bacteria, *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Streptococcus pneumoniae* were tested in the present study. Methicillin-resistant *Staphylococcus aureus* is a clinical isolate from blood samples and was acquired from the Luton & Dunstable Hospital NHS Foundation Trust, Luton, England. *Pseudomonas aeruginosa* was isolated from a clinical sample at the Aga Khan University hospital. *Streptococcus pneumoniae* was isolated from the blood culture of a pneumonia patient. *P. aeruginosa* and MRSA were grown overnight in a shaking incubator by inoculating bacterial culture in an enriched media, Luria-Bertani (LB) broth at 37 °C, whereas 5% CO₂ was used to culture *S. pneumoniae* and *S. pyogenes* in brain-heart infusion (BHI) broth

overnight at 37 °C. Although ISO 14729 requires the use of ATCC strains of bacterial organisms for testing, the bacterial strains used in this study were clinical isolates derived from human specimens and relevant to test contact lens disinfectants. *Acanthamoeba castellanii* belonging to the T4 genotype (American Type Culture Collection, ATCC 50492) was used. It is originally isolated from a patient suffering from *Acanthamoeba* keratitis. *Acanthamoeba castellanii* was cultured in a T-75 tissue culture flask in 10 mL Proteose peptone-Yeast extract-Glucose (PYG) medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose] at 37 °C as previously described (Sissons et al., 2005). Before performing assays, Fresh PYG media was added in each flask approximately 17–20 h before, to obtain amoebae in the trophozoite form. In order to obtain cysts, Non-nutrient agar (NNA) plates were seeded with trophozoites aseptically. Next, plates were kept at 30 °C in an incubator for 14 days. Following this, 10 mL sterile PBS was added to each plate and cysts were scraped off by using a cell scraper. The cyst suspension was then centrifuged at 2000 \times g for 10 min. Finally, the resultant pellet was resuspended in PBS after discarding the supernatant and cysts were counted using a haemocytometer.

2.3. Bactericidal assays

Bactericidal assays were performed to evaluate the bactericidal activity of various contact lens disinfectants against MRSA, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. International Organization for Standardization (ISO) 14729 Stand Alone Test criteria for examining contact lens disinfectants efficacy was employed as described previously (Rosenthal et al., 2002). Briefly, the optical density of bacterial broth cultures was adjusted to 0.22 at 595 nm using a spectrophotometer. Next, 10⁶ colony forming units (CFU) of bacterial cultures were added in Eppendorf tubes. Tubes were centrifuged at 12,000 \times g for 2 min to pellet bacterial CFU. The supernatant was aspirated followed by addition of 200 μ L of six different contact lens disinfectants to resuspend bacteria. Following 6 h of incubation, bacteria were enumerated by plating as described previously (Abjani et al., 2016). Bacteria incubated with PBS alone and bacteria incubated with gentamicin 100 μ g per mL were used

Table 1

List of contact lens disinfectants tested in the present study together with their ingredients, manufacturer, type of solution and Minimum recommended disinfection time (MRDT).

Solution	Ingredients	Manufacturer	Minimum recommended disinfection time (MRDT)	Type of solution
Oxysept [®]	3% Hydrogen peroxide	Abbot Medical Optics, Hangzhou	6 h	Hydrogen peroxide system
AO SEPT PLUS	3% Hydrogen peroxide	Alcon laboratories, Inc. Fort Worth, USA.	6 h	Hydrogen peroxide system
OPTI-FREE [®] PureMoist [®]	POLYQUAD [®] (polyquaternium-1) 0.001% and ALDOX [®] (Myristamidopropyl dimethylamine) 0.0006% preservatives	Alcon laboratories, Inc. Fort Worth, Texas USA.	6 h	Multi-purpose Disinfecting solution
COMPLETE RevitaLens [™]	Alexidine dihydrochloride 0.00016%, Polyquaternium-1 0.0003%, boric acid, sodium borate decahydrate, TETRONIC 904, edetate disodium, sodium citrate, sodium chloride and purified water.	Abbot Medical Optics, Hangzhou	6 h	Multi-purpose Disinfecting solution
Renu [®] fresh [™]	HYDRANATE (hydroxyalkylphosphonate), boric acid, edetate disodium, poloxamine, sodium borate and sodiumchloride, preserved with DYMED [™] (polyaminopropyl biguanide) 0.0001%	Bausch & Lomb Greenville, USA.	4 h	Multi-purpose solution
FreshKon [®] CLEAR	Sodium and Potassium chloride, Disodium Edetate, Polyhexanide, Poloxamer, Hypromellose and Sodium Phosphate Buffer	Oculus private limited, Singapore	6 h	Multi-purpose solution

as negative and positive controls. All experiments were performed several times in duplicate.

2.4. Amoebicidal assays

The amoebicidal assays were performed as previously described (Abjani et al., 2016). Briefly, contact lens disinfectants were incubated with 5×10^5 amoebae (final volume: 200 μ L) for 6 h at room temperature as per Manufacturers' instructions. In some experiments, amoebae were incubated with contact lens disinfectants containing chlorhexidine (50 μ M) and cellulase (70 units). Next, 0.1% Trypan blue was added to enumerate number of viable trophozoites by Trypan blue exclusion assay (dead cells having porous cell membrane allows Trypan blue entry and as a result cells appear blue whereas live cells remain unstained). To further confirm the viability of amoebae, amoebae were centrifuged at $2000 \times g$ for 5 min post-treatment to remove contact lens disinfectants, cellulase and residual drugs. The resultant pellet was resuspended in 200 μ L of PYG and was re-inoculated in 96-well plates for 72 h at 30 °C. Additionally, amoeba trophozoites were inoculated in Roswell Park Memorial Institute-1640 (RPMI) medium alone as a control. All experiments were conducted several times in duplicate. The data are presented as mean \pm standard error.

2.5. Cysticidal assays

Cysticidal assays were performed as described above. Briefly, cysts (5×10^4 cyst) were incubated with contact lens disinfectants, along with chlorhexidine (50 μ M) and cellulase (70 units) for 6 h at room temperature as above, followed by Trypan blue exclusion assay and enumeration of dead cells using haemocytometer counting. The viability of amoeba cysts was further confirmed using inoculating cysts, post-treatment, in fresh PYG as described above in 96-well plates for 72 h at 30 °C. For negative control, cysts were inoculated in Roswell Park Memorial Institute-1640 (RPMI) medium alone. All experiments were conducted several times in duplicate. The data are presented as mean \pm standard error.

3. Results

3.1. Bactericidal effect of multipurpose contact lens disinfectant against *P. aeruginosa* and MRSA, *Streptococcus pyogenes* and *Streptococcus pneumoniae*

To evaluate the bactericidal effects of contact lens disinfectants on *P. aeruginosa* and MRSA, assays were performed by inoculating 10^6 bacteria in 200 μ L of contact lens disinfectants. The results revealed that all six contact lens disinfectants: Oxysept®, AO SEPT PLUS, OPTI-FREE® PureMoist, COMPLETE RevitaLens, Renu® fresh and FreshKon® CLEAR exhibited potent bactericidal effects against MRSA (Fig. 1A), *P. aeruginosa* (Fig. 1B), *Streptococcus pyogenes* (Fig. 1C), and *Streptococcus pneumoniae* (Fig. 1D) within Manufacturer's Minimum Recommended Disinfection time (MRDT) and no bacterial colonies were observed. For positive controls, bacteria incubated with 100 μ g per mL of gentamicin abolished bacterial viability. When incubated alone, bacteria exhibited growth compared with the original inoculum.

3.2. Combination of cellulase and chlorhexidine improved efficacy of contact lens disinfectants against *A. castellanii* trophozoites

To determine the effects of contact lens disinfectants against *Acanthamoeba castellanii* trophozoites, amoebae were incubated with contact lens solutions. The results showed that all four multipurpose Contact lens disinfectants (without hydrogen peroxide) OPTI-FREE® pure moist®, Renu® fresh™, FreshKon® CLEAR and COMPLETE RevitaLens™ were ineffective in killing *A. castellanii* trophozoites. There was no difference between the original inoculum (5×10^5 amoebae) and the number of viable *Acanthamoeba* post-treatment, $4.29 \times 10^5 \pm 3.55 \times 10^4$, $4.07 \times 10^5 \pm 7.3 \times 10^4$, $4.07 \times 10^5 \pm 7.7 \times 10^4$ and $3.67 \times 10^5 \pm 2.0 \times 10^3$ using OPTI-FREE® pure moist®, Renu® fresh™, FreshKon® CLEAR and COMPLETE RevitaLens™ respectively (Fig. 2). Moreover, when amoebae were inoculated in fresh PYG post-treatment, viable trophozoites emerged within 24 h. In contrast, hydrogen peroxide containing disinfectants showed partial amoebicidal effects and the number of viable amoeba was $7.35 \times 10^4 \pm 3.55 \times 10^4$, $4.95 \times 10^4 \pm 5.5 \times 10^3$ for Oxysept®, and AO SEPT PLUS respectively (Fig. 2).

To determine whether the addition of cellulase and chlorhexidine at micromolar concentration improve the efficacy of contact lens disinfectants, cellulase enzyme was added at 70 μ M. The results revealed that the addition of cellulase showed no effect on the viability of trophozoites and after 6 h of incubation the number of viable amoebae was $3.28 \times 10^5 \pm 3.5 \times 10^3$. When cellulase was combined with contact lens disinfectants, OPTI-FREE® pure moist®, Renu® fresh™, FreshKon® CLEAR and COMPLETE RevitaLens™ the number of viable amoeba was $2 \times 10^5 \pm 2 \times 10^3$, $2.68 \times 10^5 \pm 4.0 \times 10^4$, $2.67 \times 10^5 \pm 1.1 \times 10^4$ and $2.66 \times 10^5 \pm 1.5 \times 10^4$ respectively but upon re-inoculation in fresh PYG, viable trophozoites emerged within 72 h. Similarly, chlorhexidine alone (up to 50 μ M) showed potent amoebicidal effects on the viability of *A. castellanii* trophozoites.

Notably, contact lens disinfectants, OPTI-FREE® pure moist®, Renu® fresh™, FreshKon® CLEAR and COMPLETE RevitaLens™ in combination with cellulase (70 units) and chlorhexidine (50 μ M) abolished viability of *A. castellanii* trophozoites within 6 h of incubation and the number was reduced to $3.85 \times 10^4 \pm 1.2 \times 10^4$, $1.50 \times 10^4 \pm 4.00 \times 10^3$, $3.10 \times 10^4 \pm 9 \times 10^3$ and $3 \times 10^4 \pm 1 \times 10^3$ respectively (Fig. 2). Post-treatment, amoebae were unable to revive in fresh PYG medium even after 72 h (data not shown). Amoebae in RPMI as negative control and in methanol as solvent control for chlorhexidine showed no amoebicidal effect and the number was $5.52 \times 10^5 \pm 6.5 \times 10^4$ and $4.01 \times 10^5 \pm 5.3 \times 10^4$ respectively.

3.3. Addition of cellulase and chlorhexidine improved efficacy of contact lens disinfectants against cysts of *A. castellanii*

Cysticidal assays were performed to evaluate the efficacy of contact lens disinfectants. The results revealed that all four multipurpose Contact lens disinfectant (without hydrogen peroxide) OPTI-FREE® pure moist®, Renu® fresh™, FreshKon® CLEAR and COMPLETE RevitaLens™ were ineffective in killing *A. castellanii* cyst (Fig. 3), and viable amoebae emerged when inoculated in fresh PYG compared with the original inoculum (dotted line). Post-treatment, viable trophozoites emerged within 72 h. Hydrogen peroxide containing disinfectants (Oxysept® and AO SEPT PLUS) showed partial cysticidal effects (Fig. 3). Cellulase

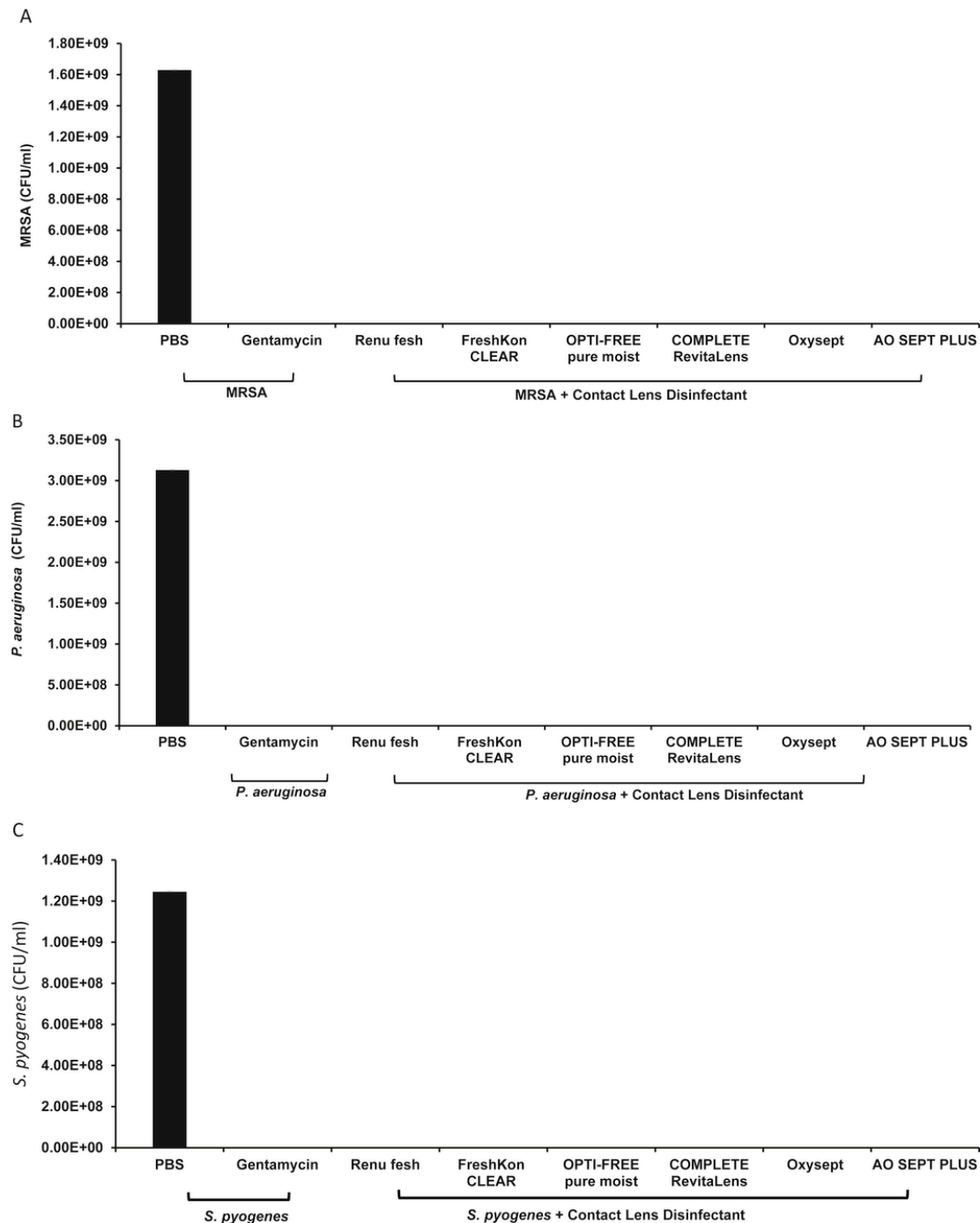


Fig. 1. Commonly used contact lens disinfectants marketed in Malaysia were tested including Oxysept (OS), AO SEPT PLUS (AO SP), OPTI-FREE pure moist (OF PM), Renu fresh (RF), FreshKon CLEAR (FKC) and COMPLETE RevitaLens (CRL) against MRSA (A), *P. aeruginosa* (B), *Streptococcus pyogenes* (C), and *Streptococcus pneumoniae* (D) using Manufacturer's Minimum Recommended Disinfection time (MRDT) as described in Materials and Methods. 100 µg/mL of gentamicin was used as a positive control, while bacteria incubated in PBS alone were used as a negative control. All six disinfectants tested abolished bacterial viability. The data is representative of several experiments performed in duplicate as expressed as the mean ± standard error.

enzyme when used at 70 µM showed no significant effect and the number was $1.72 \times 10^5 \pm 1.69 \times 10^4$. However, combination of cellulase and chlorhexidine with contact lens disinfectants abolished viability of *A. castellanii* cysts and amoebae were unable to revive when inoculated in fresh PYG (Fig. 3).

4. Discussion

Keratitis is a vision-threatening disease of the eye often associated with the use of contact lens (Mascarenhas et al., 2014). If not treated promptly, it can lead to permanent vision impairment. Improper contact lens hygiene, use of homemade saline

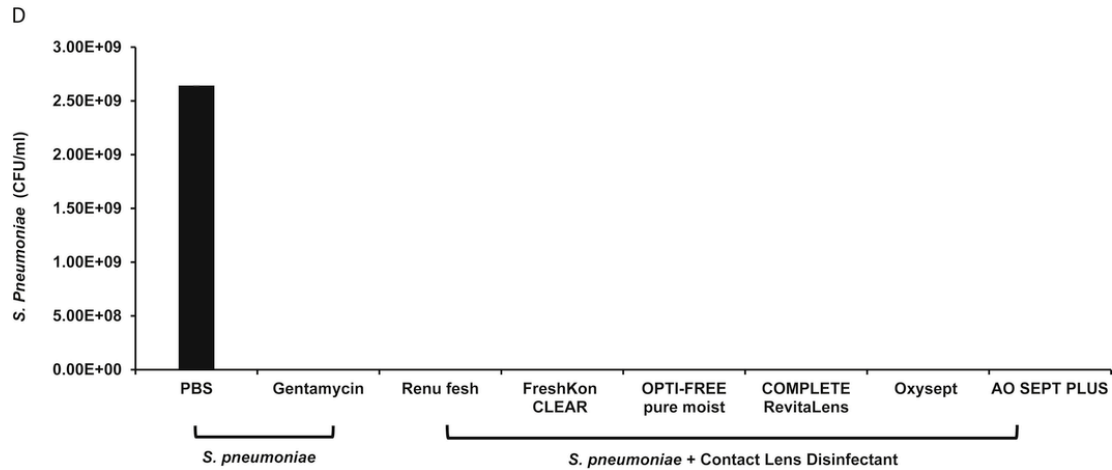


Fig. 1. (Continued)

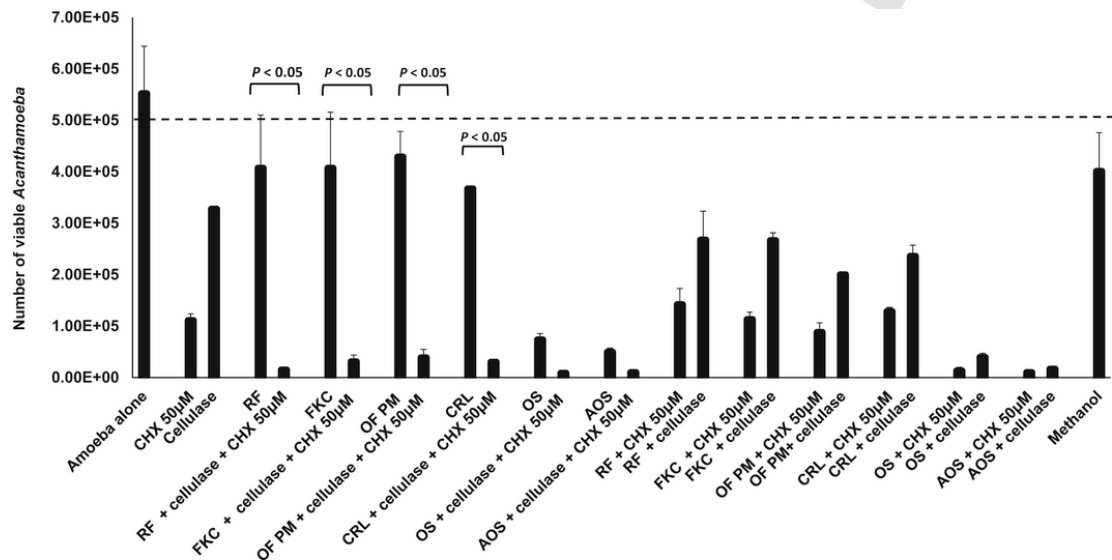


Fig. 2. Contact lens disinfectants including Oxysept (OS), AO SEPT PLUS (AO SP), OPTI-FREE pure moist (OF PM), Renu fresh (RF), FreshKon CLEAR (FKC) and COMPLETE RevitaLens (CRL) were tested against *A. castellanii* trophozoites as described in Materials and Methods. Note that none of the contact lens disinfectants destroyed amoebae effectively. However, addition of chlorhexidine (CHX) and cellulase to contact lens disinfectant abolished *A. castellanii* viability as compared to the contact lens disinfectant alone. The dotted line represents initial inoculum of trophozoites. The data is expressed as the mean \pm standard error of several experiments performed in duplicate.

solutions for cleaning and storing lenses as well as using expired disinfectants further leads to an increase risk of microbial keratitis among contact lens wearers (Stapleton et al., 2007; Stapleton and Carnt, 2012; Lorenzo-Morales et al., 2013). In this study, we determine the effectiveness of contact lens disinfectants available commercially in Malaysia against common bacterial eye pathogens and corneal parasite, *Acanthamoeba castellanii*. Our findings revealed that all contact lens disinfectants tested exhibited potent bactericidal effects against species tested. However, none of the contact lens disinfectants completely destroyed *A. castellanii* trophozoites and cyst. These findings are consistent with several recent studies indicating that marketed contact lens disinfectants are ineffective in completely destroying amoebae (Kolar et al., 2015; Padzik et al., 2014; Polat et al., 2007; Ustüntürk and Zeybek, 2014). In particular, there show limited efficacy against cysts. Chlorhexidine at 50 µM concentration destroyed amoeba within 6 h of incubation time at room temper-

ature. However, cellulase treatment had no effect on the viability of amoebae and parasites re-emerged as viable trophozoites upon re-inoculation in PYG. In contrast, a combination of chlorhexidine and cellulase together with contact lens disinfectants proved highly effective in killing both *A. castellanii* cyst and trophozoites. Our findings showed that targeting the cellulose synthesis pathway inhibits *Acanthamoeba* switching into the cyst form. This is likely due to the ability of cellulase to destroy cyst walls by degrading cellulose, allowing chlorhexidine to target the resident trophozoite within and ultimately leading to its death. This is consistent with our previous findings which showed that the addition of cellulase enzyme together with chlorhexidine enhanced the efficacy of contact lens solutions marketed in Pakistan (Abjani et al., 2016). As cellulose synthesis is limited to plants, yeast, and some bacteria, it is likely to have minimal side effects against human cells. Overall, the results obtained from our previous as well as the present study clearly demonstrate that there

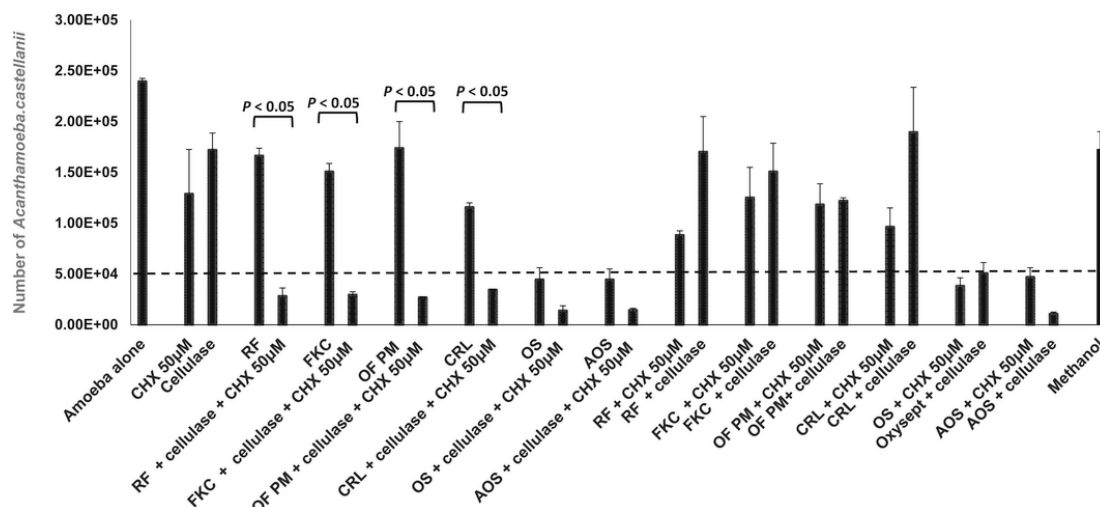


Fig. 3. Contact lens disinfectants including Oxysept (OS), AO SEPT PLUS (AO SP), OPTI-FREE pure moist (OF PM), Renu fresh (RF), FreshKon CLEAR (FKC) and COMPLETE RevitaLens (CRL) were tested against *A. castellanii* cysts as described in Materials and Methods. Note that none of the contact lens disinfectants destroyed amoebae cysts. However, addition of chlorhexidine (CHX) and cellulase to contact lens disinfectant abolished *A. castellanii* viability as compared to the contact lens disinfectant alone. The dotted line represents initial inoculum of cysts. The data is expressed as the mean \pm standard error of several experiments performed in duplicate.

is a need for an improved contact lens disinfectant against *Acanthamoeba castellanii*. As cysts are partly made of cellulose, degradation of cellulose by cellulase enzyme and chlorhexidine is a promising strategy in targeting *A. castellanii* trophozoites as well as cysts. Our findings also highlight the ineffectiveness of commercially available contact lens disinfectants in Malaysia that present a major health risk to the public. We propose that a similar strategy can be employed in the improved treatment of *Acanthamoeba* keratitis that can normally last up to a year and often complicated due to infection recurrence, however intensive research is needed over the next few years to determine the translational value of these findings.

Conflicts of interest

None to declare.

Uncited references

Lakhundi et al., 2014.

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