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In vitro effects of multi-purpose contact lens disinfecting solutions towards survivability of *Acanthamoeba* genotype T4 in Malaysia

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ABSTRACT

The incidence of *Acanthamoeba* keratitis has been increasing since the previous decades, especially among contact lens users. This infection is majorly caused by the use of ineffective contact lens disinfecting solution. Thus, this study was conducted to evaluate the *in vitro* effects of multi-purpose disinfecting solutions (MPDS) against *Acanthamoeba* trophozoites and cysts. *Acanthamoeba* genotype T4 isolated from contact lens paraphernalia and an environmental strains were propagated for trophozoite or cyst-containing culture and adjusted in final concentration of 1×10^5 cells/ml. Amoebicidal and cysticidal assays were conducted by incubating trophozoites and cysts with OPTI-FREE® Express®, ReNu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive according to the manufacturer's minimum recommended disinfectant time (MMRDT) for up to 12 h at 30 °C. Trypan blue hemocytometer-based microscopic counts determined amoebicidal and cysticidal effects. The viability of *Acanthamoeba* trophozoites and cysts was confirmed by re-inoculated them in the 1.5% non-nutrient agar plates. It was found that none of the MPDS showed amoebicidal and cysticidal effects during the MMRDT. However, OPTI-FREE® Express® demonstrated a significant differences in average cell reduction for both stages within MMRDT. When subjected to 12 h exposure, both OPTI-FREE® Express® and ReNu® Fresh™ led to significant reduction in the number of trophozoite and cyst cells. Notably, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive did appreciably improve the solution effectiveness towards trophozoite cells when incubated for 12 h. All MPDS were largely ineffective, with 100% survival of all isolates at MMRDT, while OPTI-FREE® Express® showed limited amoebicidal activity against the contact lens paraphernalia isolate, however, it was more against the environmental strains after 12 h incubation time. The commercially available MPDS employed in this research offered minimal effectiveness against the protozoa despite the contact time. Improvement or development of new solution should consider the adjustment of the appropriate disinfectant concentration, adequate exposure time or the incorporation of novel chemical elements, which are effective against *Acanthamoeba* for accelerated disinfecting and more reduction of potential exposure of contact lens users to *Acanthamoeba* keratitis.

1. Introduction

Acanthamoeba is a genus of free-living amoebae, which is prevalent in water and soil surroundings. These protozoa have also been isolated from dust, sewage, hot springs, air filters, contact lens (CL) storage cases and CL disinfecting solutions (Marciano-Cabral and Cabral, 2003; Shoff et al., 2008). *Acanthamoeba* exists in two forms including a metabolically active and more susceptible trophozoite form, and a dormant highly resistant cyst form. Several strains of the free-living amoebae could be present as facultative parasites,

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which are the causative agents of severe human illnesses. The fatal granulomatous amoebic encephalitis exists primarily in the immunocompromised patients, while the vision threatening *Acanthamoeba* keratitis (AK) is detected in the immunocompetent individuals (de Aguiar et al., 2013). The virulence of the amoebae is related to its genotype, which could be determined by sequence analysis of the 18S rRNA gene. Evolutionary studies have contributed to the identification of at least 22 genotypes of *Acanthamoeba* (T1-T22), where T4 genotype had the highest association with blinding keratitis (Tice et al., 2016).

The first documented case of AK was reported in the early 1970 s, but the rise in the incidences of AK occurred for more than 20 years around the world, including Malaysia as a result of widespread use of CL (Abd Ghani et al., 2016; Padzik et al., 2016). Provided that approximately 90% of cases of AK were related to CL use, the users of CL were faced with a higher risk of being infected (Derda et al., 2015). Although the accurate mechanism of AK related to CL was not completely grasped, the application of ineffective lens disinfecting systems, home-made saline solution, tap water and contaminated lens storage cases were frequently emphasised as crucial risk factors of AK (Moon et al., 2016). It is known that trophozoites and cysts of the amoebae may attach to the surface of the CL storage cases and the CL. The protozoans may be transmitted from the potential sources into the eyes as it happens to CL-wearing keratitis patients.

Cleaning and disinfection of contact lenses are essential to avoid lens related infection of the cornea. It is noteworthy that CL disinfection solutions are essential for safety when wearing CL. The multi-purpose disinfecting solutions (MPDS) are the most common products to disinfect lens, which are also made to clean, rinse, disinfect and store CL using a single formulation (Santodomingo-Rubido et al., 2006). The majority of these solutions comprise polyhexamethylene biguanide (PHMB) or polyquad (poly-quaternium-1) (Choy et al., 2012). Moreover, Abjani et al. (2017) highlighted that six commercially obtainable MPDS showed substantially stronger disinfection abilities against bacterial species. However, the sensitivity of *Acanthamoeba* to CL disinfecting solutions varies based on the stage of the organism (trophozoite or cyst), type and dilution of disinfecting solution and length of exposure time (Zanetti et al., 1995). This situation was caused by *Acanthamoeba* capability to change into phenotypically distinguished cyst form under severe states. Cysts have double-walled, hardy structures with the lowest metabolic activity, contributing to its resistance to harsh environmental states and biocides (Siddiqui and Khan, 2012).

Despite various research conducted to assess the impacts of diverse MPDS on *Acanthamoeba* (Heaselgrave et al., 2010; Kobayashi et al., 2011), different methodologies have created challenges in comparing these research findings and concluding the product relative effectiveness. This phenomenon was partly due to the absence of a standard approach for evaluating the effectiveness of MPDS against *Acanthamoeba* cysts and trophozoites. Based on the International Organisation for Standardisation (ISO 14729:2001) guidelines, the testing of solutions against *Acanthamoeba* is not essential. In the guidelines, the decrease in bacteria in CL storage cases is considered to be able to prevent *Acanthamoeba* growth and contamination by removing the food source of the amoebae (ISO, 2001). However, any *Acanthamoeba* left in CL storage cases may bring disadvantage to the user as no identification has been made on the lowest infective dose for infection (Beattie et al., 2003; Kobayashi et al., 2011).

In Malaysia, several varieties of MPDS are commercially available. Nevertheless, a comprehensive and objective investigation of the effectiveness of these products against *Acanthamoeba* genotype T4 have not yet been performed. Therefore, considering the growing number of users of both corrective and cosmetic CL, the purpose of this study was to evaluate the effects of four MPDS (OPTI-FREE® Express®, ReNu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive) against trophozoites and cysts of *Acanthamoeba* genotype T4 from CL paraphernalia and two environmental strains. Environmental strains were chosen as they indicated the possible causative agents of illness in a significantly common and widespread exposure.

2. Materials and methods

2.1. Acanthamoeba culture procedures

Acanthamoeba isolated from CL paraphernalia (CL54), hot spring (SLGD1) and beach water (TB5) were used in this research. All three isolates were identified as genotype T4 through the sequence analysis of the 18S rRNA gene, which is the most common genotype isolated in *Acanthamoeba* keratitis. The genome sequences of the isolates were submitted to GenBank under the accession numbers MH791020, MH790983 and MN700296 (Mohd Hussain et al., 2019, 2020). Trophozoites were grown monoxenically on a new 1.5% non-nutrient agar (NNA) (Sigma Aldrich A7002, USA) plates seeded with *E. coli* at 30 ^{0}C for three days (Eroğlu et al., 2015). The preparation of cysts was made from approximately 100 late-log-phase trophozoite cultures using 1.5% NNA medium, as previously established by de Aguiar et al. (2013). Briefly, the trophozoites were examined daily and harvested at the end of three to four days, in which the trophozoites exponentially grew. Furthermore, 2 ml of Page's amoebic saline (PAS) solution was added, followed by the harvesting of the trophozoites from the plate by gently scraping it with a sterile L shape scraper. The trophozoites were centrifuged at 3500 rpm for 10 min. The fresh preparation of trophozoite suspensions was made before they were examined. In a separate plate, cysts were acquired from cultures ageing up to two weeks (two-week-old cysts) at 30 ^{0}C. The daily examination was performed on the cultures, followed by harvesting at the end of 14 days when the microscopic examination recorded that 95% of the cells were mature cysts. The cysts were harvested as described above. The supernatants were discarded and the pellets were washed twice (3000 rpm for 5 min) by resuspension in PAS solution and stored at 4 ^{0}C for testing within 14 days. Cyst and trophozoite populations were counted using a hemocytometer. Each preparation was counted three times and the average number of these readings was used to determine the number of cells per ml. Stock solution of 5×10^5 cells/ml were prepared for the amoebicidal, cysticidal and survival assays. A total of 1×10^5 trophozoites or cysts/ml were used for the experiments (Kilvington and Lam, 2013).

2.2. Preparation of escherichia coli bacterial lawns

A single colony of *E. coli* (strain K12, American Type Culture Collection, Manassas, VA) initially grown onto a nutrient agar was selected and mixed into 40 ml of Nutrient broth (Merck Millipore, Germany). The incubation of the broth was made at 37 ^{0}C, which was then shaken at 250 rpm for one night. The *E. coli* broth was used to prepare bacterial lawns on 1.5% NNA (Sigma Aldrich A7002, USA) plates containing PAS solution to provide nutrient to the trophozoites and cysts through the incubation period of the viability experiment. The *E. coli* were plated onto 4 ml of solidified NNA prepared in a new culture plate. Gentle rotation of the plates in a circular motion was made to extend the bacterial suspension. The plates were inserted in a biosafety cabinet for 1 h at room temperature to form a smooth dense bacterial lawn of *E. coli*.

2.3. Commercial soft contact lens disinfectant solutions

The CL disinfectant solutions employed in this research, their active ingredients and manufacturers' instructions are presented in Table 1. All tested solutions were commonly marketed in Malaysia and were bought from local retailers. Fresh solutions were removed from the original wrappings and used before their stated expiration date. The examined CL disinfectant solutions represent the four most common types of multi-purpose disinfecting solutions (MPDS) for contact lens care in Malaysia. All samples were exposed to the following MPDS namely Opti-Free® Express®, Renu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive.

2.4. Amoebicidal activity of contact lens disinfectant solutions

To study the killing effects of various MPDS against *Acanthamoeba* genotype T4, amoebicidal assays were performed. Briefly, 200 μl of the calibrated trophozoite stock suspension (containing of 1×10^5 trophozoites/ml) was added to each Eppendorf tube separately. A total of 800 μl of the respective MPDS listed in Table 1 was added to each tube (total volume of 1 ml) and the samples were homogenized by vortexing. The plates were then sealed and incubated at room temperature in dark condition. Next, amoebae counts were performed using a hemocytometer after the manufacturer's minimum recommended disinfectant time (MMRDT), including 12 h s(overnight) of extended duration (Abjani et al., 2017). The viability of trophozoites was determined by using 0.4% 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). All experiments were performed in triplicate. The PAS solution was applied as the negative control, chlorhexidine (Sigma) 0.02% was served as the positive control.

2.5. Cysticidal activity of contact lens disinfectant solutions

The MPDS that were effective against *Acanthamoeba* genotype T4 trophozoites were also tested for their effectiveness against cysts. Briefly, 1×10^5 cysts/ml were pelleted by centrifugation as described above and then re-suspended in a total volume of 1 ml MPDS according to the manufacturer's instruction, as well as for up to 12 h (extended time). All experiments were performed in triplicate.

2.6. Survival assay

This method described by Buck et al. (2000), used NNA overlaid with an *E. coli* lawn to detect viable trophozoites and cysts after challenge with the test solutions. Post-treatment amoebae was re-suspended in 1 ml of PBS and centrifuged at 3500 rpm for 5 min. The supernatant was discarded and pellet re-suspended in PBS. This process was repeated three times to remove residual CL disinfectant. Subsequently, the amoebae pellet was re-suspended in 500 ml of PAS solution and inoculated on 1.5% NNA plates at 30 ^{O}C for 5 days. The plates were visualized intermittently to observe amoebic traits on the *E. coli* lawn, while the digital images were obtained using a camera-equipped stereomicroscope five days post-plating. Notably, *Acanthamoeba* genotype T4 in PAS solution alone served as negative control. Overall, all experiments were performed in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016 and SPSS software (IBM SPSS Statistics) program for Windows version 22.0 (SPSS Inc., USA). Descriptive analysis was presented as mean ± standard error to describe the effectiveness of each MPDS against test samples. For statistical comparisons, differences between groups were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey post hoc test. Differences between the MMRDT and extended disinfection time of each MPDS was determined by paired *t*-test. The value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effectiveness of contact lens disinfectant solutions against Acanthamoeba genotype T4 at manufacturer's minimum recommended disinfection time

The results revealed that none of the MPDS tested completely destroyed *Acanthamoeba* genotype T4 trophozoites and cysts as per MMRDT. However, there was a significant difference in average cells reduction between MPDS for trophozoites [$F(3, 32) = 8.588$, $p < 0.001$] and cysts [$F(3, 32) = 15.082$, $p < 0.001$], as determined by one-way ANOVA.

A Tukey post hoc test exhibited a significant pairwise differences between OPTI-FREE® Express® (62222.22 ± 20783.27 cells/ml) with ReNu® Fresh™ (83333.33 ± 11989.57 cells/ml, $p = 0.009$), Complete® Multi-Purpose Solution (91111.11 ± 7406.8

Table 1
Multi-purpose disinfecting solutions, their ingredients and manufacturer's minimum recommended disinfection time.

Product name	Contact lens type	Active ingredient (s)	Other ingredient (s)	Recommended disinfection time	Manufacturer
Opti-Free® Express®	Soft	POLYQUAD® (polydronium chloride; 0.001%), ALDOX® (myristamidopropyl dimethylamine; 0.0005%)	Sodium chloride, sorbitol, edetate disodium, boric acid, aminomethylpropanol, citrate, TETRONIC 1304	6 h	Alcon Laboratories, Inc. Fort Worth, Texas
Renu® Fresh™	Soft	DYMED™ (polyaminopropyl biguanide; 0.0001%)	HYDRANATE® (hydroxylalkylphosphonate), boric acid, edetate disodium, poloxamine, sodium borate, sodium chloride	4 h	Bausch & Lomb, Rochester, N.Y, USA
Complete® Multi-Purpose Solution	Soft	Polyhexamethylene biguanide (0.0001%), Poloxamer 237 0.05%	Water, sodium chloride, potassium chloride, sodium phosphate dibasic (heptahydrate), edetate disodium, sodium phosphate monobasic (monohydrate)	6 h	AMO Ireland
AVIZOR Unica® Sensitive	Soft	Poloxamer, Polyhexanide 0.0001% in a buffered	Sodium hyaluronate, EDTA	4 h	AVIZOR S.A Madrid (SPAIN)

2 cells/ml, $p < 0.001$) and AVIZOR Unica® Sensitive (86111.11 ± 69 72.16 cells/ml, $p = 0.003$) for *Acanthamoeba* T4 trophozoites. In contrast, Complete® Multi-Purpose Solution, ReNu® Fresh™ and AVIZOR Unica® Sensitive did not exhibit amoebicidal properties (Fig. 1).

Among the four MPDS tested, only OPTI-FREE® Express® (77777.78 ± 17340.54 cells/ml) continued to show a significant pairwise difference with AVIZOR Unica® Sensitive (95555.56 ± 950 1.46 cells/ml, $p = 0.035$) and Complete® Multi-Purpose Solution (118333.33 ± 14142.136 cells/ml, $p < 0.001$) for *Acanthamoeba* T4 cysts as per MMRDT (Fig. 1). On the other hand, Complete® Multi-Purpose Solution (118333.33 ± 14142.136 cells/ml) revealed a significant increase in cells numbers as compared to OPTI-FREE® Express® (77777.78 ± 17340.54 cells/ml, $p < 0.001$), ReNu® Fresh™ (89444.44 ± 10137.93 cells/ml, $p < 0.001$) and AVIZOR Unica® Sensitive (95555.56 ± 9501.46 cells/ml, $p = 0.005$), indicating that this solution was completely ineffective against the cysts.

3.2. Quantitative evaluation of amoebicidal and cysticidal activitity of contact lens disinfectant solutions at extended disinfection time.

For stand-alone amoebicidal and cysticidal activity of the four MPDS tested, a paired-samples *t*-test was conducted to compare the number of trophozoite and cyst cells at different time intervals. As expected, OPTI-FREE® Express® consistently shows a significant decrease in the number of cells after 12 h of exposure in both *Acanthamoeba* stages. Interestingly, when trophozoites and cysts were treated with ReNu® Fresh™ for the extended disinfection time, a significant reduction in the number of trophozoite and cyst cells were also observed with $t(8) = 4.932$, $p = 0.001$ and $t(8) = 4.438$, $p = 0.002$, respectively. Surprisingly, when trophozoites were incubated in Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive for 12 h, these solutions did appreciably improve the effectiveness towards trophozoite cells with $t(8) = 2.828$, $p = 0.022$ and $t(8) = 3.051$, $p = 0.016$, respectively (Fig. 2).

3.3. Comparative survival rates of Acanthamoeba trophozoites and cysts after exposure to multi-purpose disinfecting solutions

Table 2 shows the survival rates of *Acanthamoeba* genotype T4 trophozoites and cysts of all isolates detected in 1.5% NNA plates after each time intervals. All four MPDS were largely ineffective; ReNu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive had 100% survival of all *Acanthamoeba* isolates after MMRDT and extended disinfection time, although trophozoites were consistently more susceptible to the solutions relative to the cysts. On the other hand, OPTI-FREE® Express® had 100% survival for all *Acanthamoeba* isolates after 6 h of exposure and in one of three isolates (as trophozoites) after 12 h of contact with this solution. The most resistant isolate was generally the CL54 which was isolated from CL paraphernalia. Cysts had no effect relating to *Acanthamoeba* survival for the solutions or the time intervals.

4. Discussion

The frequency of AK could be reduced through the successful disinfecting process of CL solutions against *Acanthamoeba*. Provided that the CL use was regarded as among the crucial risk elements of AK, testing was performed *in vitro* on numerous CL care solutions for their antiamoebic effect on various *Acanthamoeba* species, strains and isolates (Ahearn et al., 2012; Boost et al., 2012). The ideal MPDS should maintain strong degree of disinfection effectiveness, provide superior cleaning and extended moisture, and stability for long-term storage to prevent toxicity from the eye (Atkins, 2006). To the best of our knowledge, this is the first *in vitro* study conducted on recent CL paraphernalia and environmental *Acanthamoeba* strains assessing the potential antiamoebic effect of selected MPDS commonly used by CL users in Malaysia. In the case of the solutions' effectiveness against trophozoites and two-week-old cysts at the concentration of 1×10^5 cells/ml, this study demonstrated that four different MPDS retailed in Malaysia were ineffective against *Acanthamoeba* genotype T4 trophozoites and cysts within the MMRDT. Furthermore, these findings are important as the 4 to 6 h period fulfilled or exceeded the manufacturer's recommended disinfection times and estimated overnight disinfection. Nevertheless, the amoebicidal activity for each MPDS tested was higher compared to cysticidal activity, which was in line with the previous studies reported by Kolar et al. (2015) and Abjani et al. (2017).

It is interesting to note that in this study, OPTI-FREE® Express® exhibited a significant decrease in *Acanthamoeba* genotype T4 cell numbers for both stages according to MMRDT (6 h) and extended disinfection time (12 h). As expected, this solution presented higher disinfection effectiveness against *Acanthamoeba* trophozoites compared to cysts. This phenomenon was observed occasionally with cysts generated with magnesium salts (Hughes et al., 2003). Based on some of the previous research, it was recorded that after 6 h of exposure, log reductions were recorded in OPTI-FREE® Express® from 2.5 to 3.82 and 0.18 to 1.986 against *A. castellanii* trophozoites and cysts, respectively (Beattie et al., 2003; Buck et al., 2005). In an independent research by

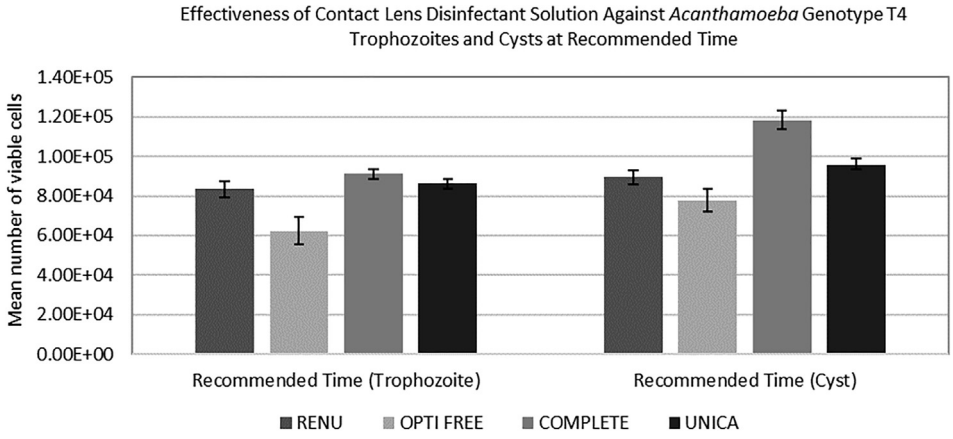

Fig. 1. Mean number of trophozoites and cysts after treatment with various multi-purpose disinfecting solutions at their manufacturers' minimum recommended disinfection time. The results are presented as the mean and standard error of two independent experiments performed in triplicate.

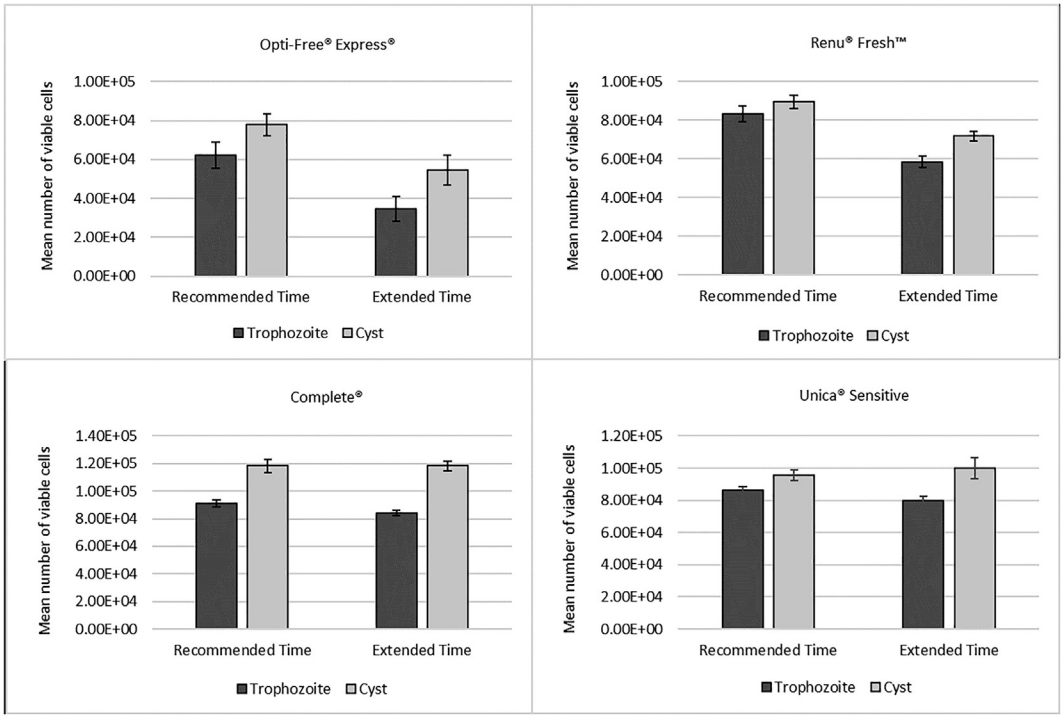

Fig. 2. Mean number of viable *Acanthamoeba* trophozoites and cysts after treatment with OPTI-FREE® Express®, ReNu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive at different time intervals. The results represent the mean and standard error of two independent experiments performed in triplicate ($p < 0.05$ using paired *t*-test).

Table 2
Survival rates of *Acanthamoeba* genotype T4 from different isolates (as trophozoites or cysts) after exposure to multi-purpose disinfecting solutions.

Product name	Isolate	Exposure time				
		Recommended disinfection time		Extended disinfection time		Overall Survival
		Trophozoite	Cyst	Trophozoite	Cyst	
Opti-Free® Express®	SLGD1	100% (3/3)	100% (3/3)	33.3% (1/3)	100% (3/3)	
	TB5	100% (3/3)	100% (3/3)	33.3% (1/3)	100% (3/3)	88.9% (32/36)
	CL54	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
Renu® Fresh™	SLGD1	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
	TB5	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (36/36)
	CL54	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
Complete® Multi-Purpose Solution	SLGD1	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
	TB5	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (36/36)
	CL54	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
AVIZOR Unica® Sensitive	SLGD1	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	
	TB5	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	100% (36/36)
	CL54	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	

Kilvington (1998) with various species of *Acanthamoeba* including *A. castellanii* and *A. polyphaga*, again OPTI-FREE® Express® achieved log reductions in viability of approximately 4 for trophozoites and 2 to 3 for cysts after the MMRDT. Notably, Alam-Eldin and Aminou (2014) found that this MPDS could destroy all trophozoites within 2 h before the MMRDT at the concentration of 5×10^3. Compared to other MPDS against *Acanthamoeba* genotype T4 trophozoites and cysts, the higher disinfection effectiveness of OPTI-FREE® Express® might be attributed to the dual disinfection system in MPDS, POLYQUAD® (polidronium chloride) 0.001%, and ALDOX® (myristamidopropyl dimethylamine) 0.0005%. POLYQUAD® was recorded with major antibacterial activity, while ALDOX® was found to have both antiamoebic and antifungal activity (Schuster et al., 2003). Furthermore, OPTI-FREE® Express® comprised a ten fold higher amount of polydronium chloride compared to polyquaternium/alexidine-based or polyquaternium/PHMB-based (Kolar et al., 2015). On the other hand, Mowrey-McKee and George (2007) demonstrated that this disinfectant solution exhibited a restricted amoebicidal effect after the suggested disinfection duration of 6 h upon being examined against a strain of *A. castellanii*, leading to 0.5 and 2.5 log reductions for cysts and trophozoites, respectively. Besides, Ustunturk and Zeybek (2014) found that OPTI-FREE® Express® achieved total destruction of trophozoites before the MMRDT but it had limited cysticidal activity. The differences between the findings of these research were attributed to the contrasts in the applied methodologies, including the cyst production, organism strain, neutralisation of the test solution, inoculum preparation, quantification method, recovery and determination of the survivor viability (Buck et al., 2005; Anger and Lally, 2008).

The present study also observed a highly encouraging trend in which ReNu® Fresh™ containing DYMED™ or polyaminopropyl biguanide (PAPB 0.0001%) destroyed trophozoites and cysts of *Acanthamoeba* after 12 h of incubation time, although they remained viable during the manufacturer's minimum recommended disinfection time. Meanwhile, Polat et al. (2007) reported

that ReNu solution could destroy the *Acanthamoeba* trophozoite after 6 h incubation time. A similar finding was also observed by Padzik et al. (2014), who revealed that the most significant amoebostatic effect occurred after being exposed to ReNu solution for 24 h, which was a notably longer duration compared to MMRDT (4 h). This element might be strengthened by the chelating agent HYDRANATE® in ReNu® Fresh™, which could justify the improved cysticidal and trophozoiticidal actions of this MPDS in comparison to those of other solutions with 1 ppm polyhexamethylene biguanide (PHMB). The fast chelation of the calcium ions outside the cell accelerated the leakage from the inside of the cell, resulting in disruption of protein and permanent cellular damage. A different hypothesis was the incorporation of sodium borate and boric acid in the formulation of ReNu® Fresh™, which was found to strengthen the PHMB process by two-fold (Khunkitti et al., 1996). In contrast, a study in Egypt reported that ReNu® MultiPlus (using PAPB as the disinfectant) could destroy the trophozoites within 2 h before the MMRDT (Alam-Eldin and Aminou, 2014). Similarly, Beattie et al. (2003) and Kobayashi et al. (2011) found that ReNu® MultiPlus was effective in the eradication of *Acanthamoeba* trophozoites within the MMRDT. There was no clarity about the factors of the different killing abilities of contrasted disinfection brands even though all solutions comprised similar concentration of the preservative (0.0001% PAPB). There are controversial reports on the effect of PAPB against *Acanthamoeba* to be found in the literature and moreover, the concentration of PAPB in ReNu contact lens solutions has been changed during the past years (Hiti et al., 2006). The present findings indicated the importance of a suitable antiamoebic agent concentration in ReNu® Fresh™ for its effectiveness against *Acanthamoeba* trophozoites and cysts when being exposed for 4 h. The cysticidal activity of CL disinfectant solution was directly proportional to the soaking time of the organisms in the solution (Wanachiwanawin et al., 2009). Therefore, an appropriate concentration of active amoebicidal ingredients and an sufficient exposure time are necessary for effective killing of both *Acanthamoeba* stages.

Polyhexamethylene biguanide (PHMB), which was produced as an environmental disinfectant and identified Baquacil by Zeneca Pharmaceuticals was found to have cysticidal and amoebicidal activity against several *Acanthamoeba* strains (Marciano-Cabral and Cabral, 2003). Some research examined the minimum cysticidal concentration (MCC) and the minimum trophozoite amoebicidal concentration (MTAC) of PHMB. Larkin et al.'s (1992) study on the *in vitro* susceptibilities of corneal isolates of *Acanthamoeba* to a wide range of drugs recorded that the MTAC of PHMB amounted to 0.87 μg/ml (range of 0.49 to 1.49 μg/ml for five isolates), while the MCC amounted to 2.11 μg/ml (range of 0.97 to 3.9 μg/ml for five isolates) after being exposed for 48 h. Similarly, the study by Hay et al. (1994) on corneal isolates of *Acanthamoeba* found that the MTAC of PHMB amounted to 1 μg/ml, while the MCC amounted to 3 μg/ml after being exposed for 48 h. It was unexpected that in this study, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive which contains 0.0001% of PHMB were able to kill trophozoite form. However, both solutions were ineffectively killed cysts after 12 h incubation time. A literature survey revealed that the current study was the first study to assess the effectiveness of AVIZOR Unica® Sensitive against *Acanthamoeba* genotype T4 trophozoites and cysts. Earlier study carried out by Polat et al. (2007) reported that trophozoites were completely destroyed after 4 h and cysts after 12 h of exposure with AVIZOR Aqua Soft Comfort, Elegance® (containing PHMB 0.0002%). A similar observation has been discovered from the use of Meni Care Plus (storage of rigid gas permeable contact lenses) with PHMB 0.0005%, completely destroyed the cysts of all strains after 8 h of exposure (Hiti et al., 2006). This observation might illustrate the ineffectiveness of overnight soaking with solutions less concentrated than 0.0005% PHMB against two-week-old cysts. Moreover, the quantitative analysis performed by Kolar et al. (2015) using the live cell imaging confirmed that the PHMB-based solution had the lowest effectiveness, as indicated through a significantly delayed response with rounding of the *Acanthamoeba* cyst cells and minimal death.

This study used three different *Acanthamoeba* strains, all of which belonged to genotype T4 but were isolated from different sources (CL paraphernalia, hot spring and beach water). Notably, differential responses were found among the different isolates to various MPDS. Overall survival of 33.3% for OPTI-FREE® Express® solution (from environmental strains) and 100% survival for ReNu® Fresh™, Complete® Multi-Purpose Solution and AVIZOR Unica® Sensitive solutions against *Acanthamoeba* trophozoites were recorded after 12 h incubation time. This finding was consistent with the report from Shoff et al. (2007), which showed that three isolates with the highest susceptibility to MPDS were environmental strains (isolated from Florida beach) with T4 genotype. Similarly, de Aguiar et al. (2013) observed that only a swimming-pool isolate (PT5) showed susceptibility to two solutions throughout the tested time intervals. One isolate that recovered from CL paraphernalia in the current study was the strain with the highest resistance due to its survival in all the solutions tested within the MMRDT and extended disinfection time. Despite the lack of clarity in the factor of the variation in the findings, it was possibly due to inherent contrasts in the strains isolated from various sources. This situation might be attributed to the hot spring and beach water, which were not exposed to any chlorinated material or toxic chemicals in the environment. Therefore, they were more susceptible to disinfectant systems. Numerous research demonstrated that various effects of disinfectants against *Acanthamoeba* were present between different strains and morphological groups (Hiti et al., 2002, 2006). This research finding implied that these abilities might vary between the strains under the same genotype. In presenting more comprehensive information about the conditions of the CL solutions, advanced research using more strains including clinical and other environmental strains are necessary.

A very critical point shown in the present study was the lack of effectiveness against *Acanthamoeba* two-week-old cysts of all tested solutions for the storage of soft CL, in which cysts were still viable after extended disinfection time. Comparable findings were reported by Kobayashi et al. (2011), Lakhundi et al. (2014) and Siddiqui et al. (2015), who found that none of the CL solutions tested in their study were able to destroy two-week-old cysts after 12 h of exposure including the one-step hydrogen peroxide solution. Therefore, it was indicated that hydrogen peroxide solutions and MPDS did not offer sufficient protection against mature cysts. Upon comparison between the impacts of disinfectant solutions against one and two-week-old cysts, the two-week-old cysts were recorded to have high resistance to all solutions, while the one-week-old cysts had equal or higher susceptibility to the solutions compared to trophozoites (Kobayashi et al., 2011). Essentially, two layers are present in the wall of the mature cyst, namely an outer wrinkled ectocyst created from protein and an inner thick, polygonal, stellate, triangular or round endocyst mainly comprising cellulose, which has a high resistance to physical and chemical agents (Johnston et al., 2009). Meanwhile, research by Kilvington and Anger (2001) indicated that immature cysts had higher sensitivity to disinfectants compared to mature cysts due to the inadequate additional cell wall material after prolonged incubation in PYG medium. The presence of complete cysts eradication or vice versa after being exposed to the examined MPDS was a crucial matter. Notably, surviving cysts in CL cases are able to excyst and multiply, initiating infection of the eye.

5. Conclusion

In conclusion, it was indicated from the discussed findings that the protective effect of the commonly used MPDS against *Acanthamoeba* genotype T4 was insufficient within a reasonable time frame. This knowledge could assist the manufacturers to improve the appropriate disinfectant concentration and adequate exposure time for effective elimination of different stages of this protozoa. The research also proposed a different method to active ingredient compositions by incorporating cellulase (50 units) and chlorhexidine (30 mM) for 6 h minimum disinfection time when pathogens such as *Acanthamoeba* became an issue for CL users as this chemical elements were able to rupture the double-walled resistant cyst. Provided that inefficacious MPDS is highly harmful to public health, these results should be strongly concerned among the health authorities and manufacturers of the CL disinfection solutions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

The concept study was conducted and designed by TSA, MKAG, NAK and RS. RHMH and WNA performed the samples collection and laboratory experiments. TSA, MKAG and RHMH were responsible for the analysis and interpretation of the data. TSA and RHMH wrote and drafted the manuscript. NAK, RS, MKAG and WNA reviewed and edited the manuscript, and then gave final approval of the version to be published. All authors read and approved the final manuscript.

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