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Extended disinfection time reduces bacteriostatic efficacy of contact lens cleansing solutions against bacterial contaminants

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ABSTRACT

Bacterial keratitis is a common eye infection case of contact lens users worldwide. This eye infection is caused by exposure to bacterial contaminants that grow due to unhygienic practices and irregular disinfection of the contact lens and its cases. This study has shown that *Staphylococcus aureus* and *Staphylococcus epidermidis* were among the common bacterial contaminants on contact lens cases. 16S rRNA sequencing of unknown bacterial isolates revealed the presence of other bacterial species such as *Escherichia vulneris* and *Bacillus subtilis*, a gram negative and a gram positive bacterium, respectively.The bacteriostatic efficacy of two commonly used multipurpose disinfecting solutions was investigated in this study. It was observed that bacteriostatic efficacy of the two disinfecting solutions was reduced as observed in the increased colony forming units on 24 hours and 48 hours extended disinfection time. Moreover, disinfection of contact lens cases without hand washing significantly increased the number of colony forming units as revealed by Paired T-test at 5% level of significance.

Keywords: Mannitol Salt Agar, 16S rRNA, *Staphylococcus aureus*, *Bacillus subtilis*, contact lens cases

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INTRODUCTION

There are approximately 125 million contact lens users across the globe (Morgan *et al*., 2011; Nzeako and Al-Sumri, 2011). Among all the contact lens accessories, the storage cases were found to be most frequently and heavily contaminated with bacteria, fungi and protozoa (Wu *et al*., 2010). Bacterial contamination on contact lens cases predisposes users in acquiring bacterial keratitis which can lead to permanent vision loss (Bourcier *et al*., 2003; Wu *et al*., 2010). Common examples of bacteria reported to cause ocular infections include *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Pens *et al*., 2008; Eltis, 2011). These bacteria which are ubiquitous are reported to be resistant to dilute solution of disinfectants. Keratitis associated with these bacteria was reported to be harder to treat because it displays multiple resistance to antibiotics (Mohammadinia *et al*., 2012).

Prevention of bacterial contamination of contact lens and storage cases can reduce the risk of developing ocular infections. This study provides information on the bacterial contaminants on poorly managed contact lens storage cases. This study also investigated the effect of prolonged disinfection time on the bacteriostatic efficacy of two commonly used multipurpose disinfecting solutions in the Philippines.

MATERIALS AND METHODS

Bacterial Isolation and Quantification

Sterile cotton swabs were used to sample bacterial contaminants on contact lens cases. A sterilized paper mold with a cut out area of 1 cm^2 was used to standardize the surface area sampled. Swab samples were inoculated into test tubes in replicates which contained 10 ml 0.1 % peptone water, and were incubated at 37°C for 20 minutes. An inoculum of 0.1 ml from the peptone solution was transferred to a nutrient agar (NA) plate in replicates using a micropipette and was incubated at 37°C for 48 hours. Control plates were made to assure sterility of cotton swabs used. Colony forming units (CFU) were counted manually and were recorded (Enriquez *et al*., 1995).There were four time intervals during the counting of CFUs, first at Manufacturer's Recommended Disinfecting Time (MDRT), four hours MRDT for EO Flex Wear Solution (Neo Vision) and six hours MRDT for OPTI-FREE Pure Moist (Alcon). Second, after 24 hours extended disinfection time, third after 24 hours disinfection time without hand washing and lastly after 48 hours extended disinfection time. Paired T-test at 5% level of significance was used to determine whether there is significant increase in CFU over time. P-values less than 0.05 were considered statistically significant.

Bacterial Identification using Mannitol Salt Agar

Mannitol Salt Agar (MSA), a selective and differential media, was used to isolate *Staphylococcus* sp., a common commensal of the skin, hands, nose and face which can infect the eyes during hand to eye contact (Mohammadinia *et al*., 2012; Panthi *et al*., 2014). The high amount of salt (7.5% NaCl) in the media inhibits the growth of other gram positive and gram negative bacteria but allows the growth of *Staphylococcus* sp., which could tolerate high osmotic stress (Shields and Tsang, 2013). In addition, phenol red in the media acts as a pH indicator. The media will show a color change from red to yellow when mannitol is hydrolyzed by the growth of *S. aureus*. This property of the media aids in the differentiation between *S. aureus* and *S. epidermidis* which are mannitol-fermenting and mannitol non-fermenting, respectively. Gram-staining was performed to record cell shape and gram identity of isolated bacterial contaminants.

Bacterial Identification using 16S rRNA Analysis

Glycerol stock of unknown bacterial contaminants on NA plates were sent to MACROGEN Korea for 16S rRNA sequencing. For the preparation of the glycerol stock, pure cultures were transferred in an Erlenmeyer flask containing 25 ml of nutrient broth and were incubated for 24 hours at 37°C in an orbital incubator mixer (MRC ORBITAL SHAKER INCUBATOR). An inoculum of 0.1 ml was taken and transferred using a micropipette into a microcentrifuge tube and was centrifuged at 15000 rpm (15°C) for 10 minutes. This was done repeatedly until about one-fourth of the microcentrifuge tube was filled. Glycerol, 30%, was added into the microcentrifuge tube

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before it was sealed with parafilm. 16S rRNA sequences obtained were analyzed using Basic Local Alignment Search Tool (BLAST) in the National Center of Biotechnology Information database (NCBI).

RESULTS AND DISCUSSION

The high incidence of bacterial contamination on contact lens storage cases is attributed to improper handling and infrequent cleaning which permits the formation of biofilms (Pens *et al*., 2008; Rahim *et al.,* 2008). *S. aureus* and *S. epidermidis* were isolated using MSA media. These are anaerobic, gram positive bacteria. It was also reported that *S. aureus* is responsible for most human ocular infections (Rhem *et al.*, 2000). The fermentation of mannitol in the media as shown by a change in color indicated the presence of *S. aureus*, whereas the absence of color change in the MSA plate indicated the presence of *S. epidermidis* (Figure 1). Gram staining of isolates growing on MSA media revealed staphylococcus cell arrangement and gram-positive identity. 16 SrRNA sequence analysis of unknown isolates revealed bacterial species such as *Escherichia vulneris* (AB758355), *B. subtilis* (KC443103 and AY913755), *B.* cereus (HM209766) and *B.* humi (JX276537). Most of the sequences obtained showed high similarities (94-99%) using BLAST homology search (Table 1).In the study of Gopinathan and company (1994), gram negative bacteria were observed as common causative agents of ulcerative keratitis. Several bacterial species were also reported to contaminate contact lens storage cases. Among these are *Pseudomonas aeruginosa*, *Enterobacter* sp., *Klebsiella* sp., *Alcaligens* sp. and *Serratia* sp. (Bharathi *et al*., 2007).

Table 1: BLAST result of 16 SrRNA from unknown bacterial contaminants isolated on poorly managed contact lens cases.

Identity	GenBank Accession number	Group	Homology (%)
Escherichia vulneris	AB758355	Proteobacteria	99
Bacillus subtilis	KC443103	Firmicute	99
Bacillus humi	HM209766	Firmicute	93
Bacillus cereus	JX276537	Firmicute	99

Table 2: Colony forming units (CFU/cm^2) recorded on four time intervals.

Disinfecting Solution	MRDT* (CFU/cm^2)	24 Hours (CFU/cm^2)	48 Hours (CFU/cm^2)	24 Hours Without hand washing
EO Flex Wear Solution	100^a	2500^a	4200^a	71900^b
	300^a	1200^a	2100^a	34700^b
	100^a	700^a	1100^a	30400^b
	200^a	500^a	600^a	4100^b
OPTI-FREE PureMoist	200^a	300^a	1100^b	14200^b
	100^a	200^a	800^b	13900^b
	200^a	100^a	500^b	37600^b
	100^a	200^a	400^b	3200^b

* Manufacturer's Recommended Disinfection Time, EO Flex Wear Solution= four hours, OPTI-FREE PureMoist= six hours. Values with the same superscript have no significant difference based on Paired T-test at 5% level of significance.

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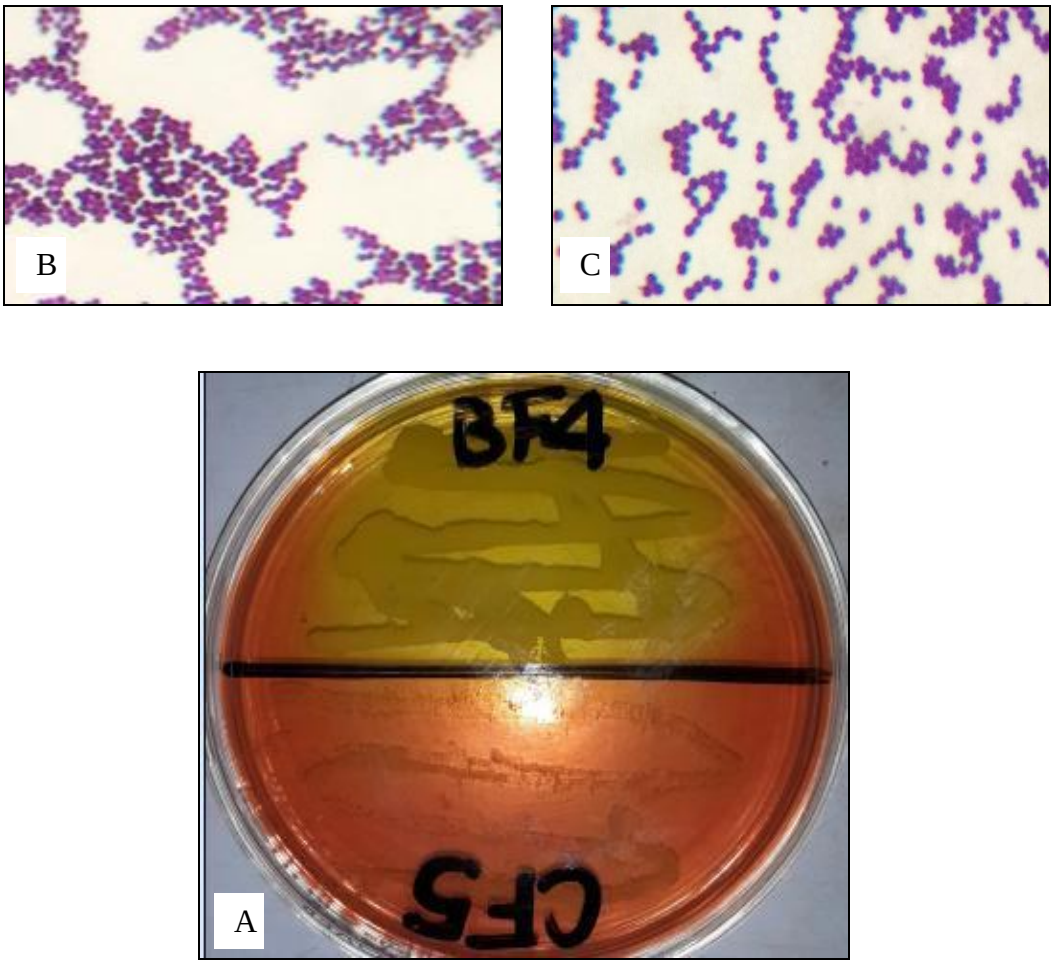

Figure 1: (A) *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on Mannitol Salt Agar (MSA) for 24 hours at 37° C.

Note: Isolate BF4, identified as *S.aureus*, due to hydrolysis of mannitol in the media, whereas isolate CF5 was identified as *S. epidermidis* due to no hydrolysis. Gram staining showed gram-positive cocci, *S. epidermidis* (B) and *S. aureus* (C) viewed under light microscope (LEICA) using oil immersion objective.

The active ingredients in the two commercial disinfectant solutions investigated in this study are quaternary ammonium compounds (QAC) (Rosenthal *et al*., 2000; Lipener, 2009; Carmona-Ribeiro and Carrasco, 2013; Iguban *et al*., 2013). The mode of action of these cationic agents is through interaction with phospholipid components in the cell membrane, which causes membrane distortion and protoplast lysis through osmotic stress (Carmona-Ribeiro and Carrasco, 2013). This study has shown that the bacteriostatic efficacy of the two disinfecting solutions decreases when used beyond the recommended disinfecting time (Table 2). Prolonged incubation time of disinfecting solutions extending to 24 hours and 48 hours had shown increased growth of bacterial colonies although it has no significant difference in number of CFUs from MRDT (EO Flex Wear Solution-24 hrs-p=0.1098, 48 hrs-p=0.0623; OPTI-FREE PureMoist- 24 hrs-p=0.3910) (Table 2). Extending the disinfection time to 48 hrs using OPTI-FREE PureMoist, however, revealed significant increase in the CFUs (p=0.0351).Moreover, disinfecting contact lens cases without hand washing has significantly increased the CFUs (p=0.048). This was also reported by Khan *et al*., (2013), that there is increased incidence of eye infection among users that does not wash their hands during the disinfection of their contact lenses. Proper hand washing and strict compliance of MRDT will decrease

the likelihood of bacterial contamination. The use of multipurpose disinfecting solutions for extended period of time should be avoided to prevent occurrence of bacterial contamination on contact lens storage cases.

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