Acta Protozool. (2011) 50: 33–41
http://www.eko.uj.edu.pl/ap

ACTA
PROTOZOOLOGICA

Tolerance of Naked Amoebae to Low Oxygen Levels with an Emphasis on the Genus *Acanthamoeba*

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Summary. Amoebae feed on attached bacteria within, and below, bacterial biofilms where they experience reduced oxygen levels. The implications of this were examined by comparing the migration (an index of growth) of thirteen strains of *Acanthamoeba* and five species of naked amoebae grown under microaerophilic and aerobic conditions. All amoebae replicated well under both conditions and twelve isolates migrated significantly faster under low oxygen. Only one isolate, *Vannella* sp., migrated further (presumably grew faster) under aerobic conditions. The data show most amoebae prefer low oxygen as befits the biofilm habitat. Interestingly, the eleven acanthamoeba strains that replicated faster under microaerophilic conditions were all T4 genotypes and included four strains isolated from patients with amoeba keratitis (AK) infections. This genotype is most frequently found in AK cases and it is suggested that strains of *Acanthamoeba* capable rapid growth in a biofilm of a poorly cleansed contact lens may be an important factor in the development of an effective infective dose when placed on the cornea.

Key words: Amoebic Keratitis, microaerophilic, naked amoebae, migration, biofilms.

INTRODUCTION

Naked amoebae have been shown to be numerically important in a wide range of habitats although little is known about their true ecological role. It has been postulated that amoebae feed on tightly associated surface bacteria and effectively graze a unique niche (Rogerson and Laybourn-Parry 1992) distinct from other protists, the heterotrophic flagellates and ciliates. This can be viewed in laboratory cultivation where amoebae attach firmly to the base of culture dishes amid dense biofilms of bacteria. In nature they have an obligate surface-associated life-style different from the other transient micro-grazers (Pickup *et al*. 2007). Under these habitat conditions, they surely must experience reduced oxygen tension as they migrate within the metabolically active bacterial film. As Turner *et al.* (1997) pointed out, given their range of habitats it is inevitable protozoa will encounter anaerobic conditions when O_2 is consumed by the many organisms in their vicinity. The ability of protozoa to tolerate low oxygen levels has been known for many years and most early studies focused on how long protozoa can survive without

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oxygen (e.g. Kitching 1939). However, the possibility that some species of amoebae prefer, or grow better, under low oxygen levels, perhaps reflecting their habitat conditions, has not been addressed, at least in the case of amoebae. On the other hand, the ecological behavior of planktonic ciliates has been studied and there are excellent examples of ciliates actively seeking out microaerophilic zones close to the oxic-anoxic boundary where they can out-compete metazoan competitors and predators (Finlay and Esteban 2001).

Understanding the oxygen tolerances of amoebae within biofilms is ecologically interesting and has applied implications for opportunistically pathogenic amoebae. In this regard, acanthamoebae are arguably the commonest type of amoeba to be isolated from soil habitats (Page 1988), a feature that attests to the ubiquity of this genus. The abundance of acanthamoebae make them important grazers of bacteria in soils but this free-living amoeba can also invade the central nervous system of immuno-suppressed individuals and cause granulomatous amoebic encephalitis (Marciano-Cabral and Cabral 2003) and the sight-threatening condition amoebic keratitis (AK) when isolates invade the cornea of contact lens wearers. The latter disease is more common but still rare with an estimated 0.33 cases per 10,000 lens wearers (Seal 2003). Factors that promote invasion of the eye are largely unknown although most agree that ineffective lens disinfection is a key factor (Devonshire *et al.* 1993) since this can allow microbial contaminants to accumulate. Co-contamination of a lens care system with bacteria and amoebae can lead to a large inocula of *Acanthamoeba* on the lens surface and hence the cornea (Gray *et al.* 1995). Gray's study looked at microbial contamination of contact lens cases and found that 81% of cases examined were contaminated with microbes, predominately bacteria. Moreover, the adherence of microbial biofilms onto plastics and their relative resistance to antiseptics has been reported (Wilson *et al.* 1990, Anwar *et al.* 1992). More recently, Beattie *et al.* (2006) showed that acanthamoebae will attach to the new first and second generation silicon hydrogel lenses and that the presence of a bacterial biofilm can be an important factor in attachment. If indeed the presence of a microbial biofilm, either on a lens surface or in the lens cleaning case, is a parameter in the etiology of the disease, the behavior of amoebae within the biofilm is important. If amoebae remain active at the lower oxygen regimes within the biofilm, if the bacteria are palatable, and amoebae are afforded protection from disinfectants (Shoff *et al.* 2007a) then strains of acanthamoebae capable of replicating under these conditions would have the potential to generate a significant population of trophic cells that is more likely to constitute an infective dose when transferred to the eye.

The present study set out to consider whether amoebae were disadvantaged or advantaged by being in low oxygen concentrations by presuming such conditions approximated those found within an active bacterial biofilm. The study considered whether oxygen concentration is a possible factor that should be considered when trying to understand why some acanthamoebae can cause amoebic keratitis. This was assessed by comparing the responses of 13 strains of acanthamoebae, all of known genotype and source (i.e. corneal isolates and environmental isolates). The study also considered whether acanthamoebae were unique, within the naked amoebae, in their response to low oxygen by testing the responses of five different genera.

MATERIALS AND METHODS

Amoebae isolates and cultivation methods

Most of the *Acanthamoeba* strains tested were from stocks held by the authors AR and WT. Their genotypes were known (Booton *et al.* 2002, 2004) as were their sources and year of isolation. Strain P 120 was isolated in 2000 from kitchen tap-water in Hong Kong (T3), and strain TIV (T5) from tap-water in Florida (2002). All other strains were T4 genotypes: strains FLA 1, 2, and 9 were from Florida beach sand (isolated 2002), strain MS26 was isolated in 2006 from tap-water in Florida and strain DS was isolated in 2002 from beach sand in Scotland. Strains PI, P6, P91 and P209 were all from corneal scrapes of patients with amoebic keratitis (approximate date of isolation 2000). Strains FSP1M and FSP2M were isolated in 2009 from benthic mud (approx 1 cm deep) in a shallow eutrophic pond on the campus of Fresno State University. It was presumed that these isolates were recovered from microaerophilic mud either as cysts or trophic amoebae. These two isolates were both T4 types as determined by genotyping methods given in Booton *et al.* (2002). Sequences were deposited in GenBank and are available through accession numbers HQ637490 (for FSP1M) and HQ637491 (for FSP2M).

All amoebae were routinely cultured on fresh non-nutrient agar (NNA) plates streaked with *Escherichia coli* as detailed by Page (1988). Plates were incubated in the dark at 20°C. When the *E. coli* prey were exhausted, a small block of agar containing about 50 cells was dissected from the plate and placed at the start of a fresh bacterial streak on a new NNA plate.

To determine whether the migration (an index of growth) of acanthamoebae under low oxygen was genus specific, other genera were isolated in 2009 from the water column by inoculating small drops of water (ca. 50 µl) onto a non-nutrient agar plate streaked with *E. coli* bacterial prey. Clonal cultures were established by dissecting out a small block of agar containing a single cell and inoculating onto fresh media. Strain FSP1A (Fig. 1a) was isolated from the water column of the small eutrophic pond on Fresno State campus. This strain was generally 25 to 30 µm in length, markedly eruptive during locomotion with occasional trailing uroidal filaments. In the absence of molecular identification it is designated as unidentified vahlkampfiid species 1. Pond amoebae 1, 2 and 3 were all isolated from the water column of a larger pond on the Fresno State campus. Pond strain 1 (Fig. 1b) was identified as *Vannella* sp. and was typically 40 to 55 µm in length. Pond strain 2 (Fig 1c) was 25 to 35 µm in length and was less flattened than classic vannellids with several small cytoplasmic crystals, a hyaloplasm with an anterior crescent and frequent extensions from the posterior. This strain is referred to as a thecate-like amoeba. Pond strain 3 (Fig 1d) was identified as *Saccamoeba* sp. based on its limax appearance (up to 80 µm in length), steady locomotion, uroid, reduced hyaline cap and numerous conspicuous cytoplasmic crystals. The Ohio River isolate (Fig. 1e) was from West Virginia and was a markedly eruptive amoeba, typically 20 to 25 µm in length. It was limax (i.e. tubular) in shape and quite distinct from the other eruptive strain (FSP1A). It also differed in the lack of trailing uroidal filaments. It is identified as vahlkampfiid species 2. While these identifications fall short of being named species, they do represent 5 different morphospecies (*sensu* Finlay and Esteban 2001) and serve for comparative purposes with the 13 strains of *Acanthamoeba.*

Migration of amoebae under microaerophilic and aerobic conditions

Amoebae were cultured in air and under microaerophilic conditions using a BD GasPak EZ generating container system (Becton, Dickinson and Co, MD, USA). This system allows for the growth of microaerophilic organisms when used with a Campy System Sachet that produces a microaerophilic atmosphere within 2 h with between 6 to 16% oxygen, and 2 to 10% carbon dioxide within 24 h. In each case, plates of a strain were replicated 15 times and incubated at 23°C either in air or under microaerophilic conditions. Culture conditions were similar to those used to maintain stocks, namely NNA plates streaked with *E. coli* bacterial prey. However, care was taken in the following areas.

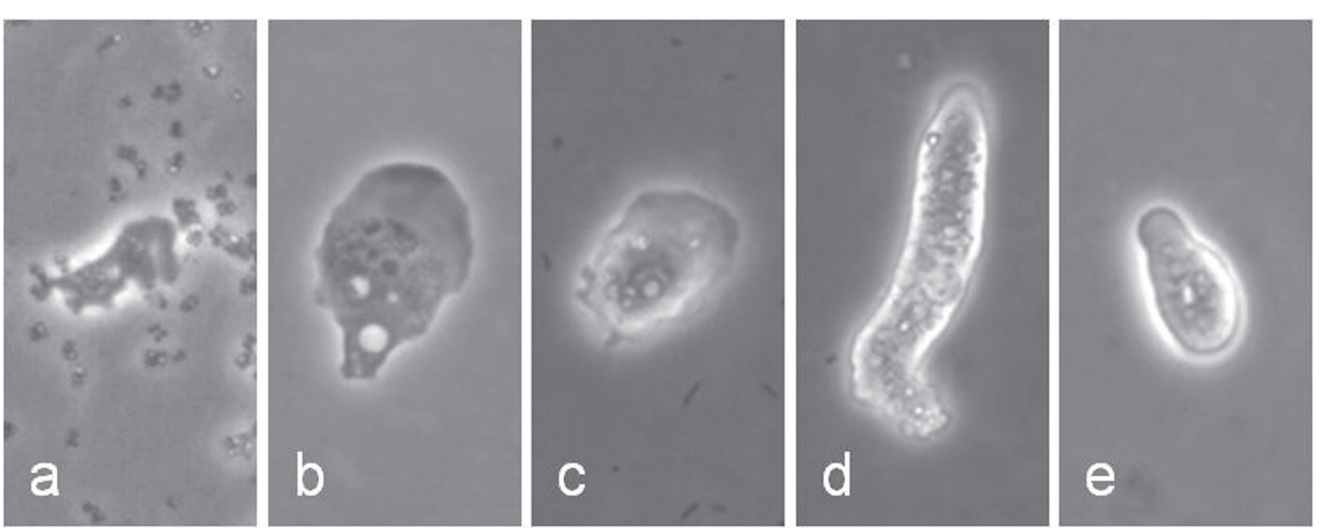

Fig. 1. Photomicrographs of the 5 isolates of amoebae (non-acanthamoebae) used in the migration experiments. **a)** strain FSP1A, length 25 µm; **b)** pond strain 1, length 45 µm; **c)** pond strain 2, length 30 µm; **d)** pond strain 3, length 65 µm. E) Ohio river strain, length 24 µm.

The density of the bacterial streak was the same in all cases, only exponentially growing *E. coli* were used, and amoebae came from recent cultures (sub-cultured 5 days previously). The block of agar with cells used to inoculate the experimental plates was taken from the edges of the bacterial streak to ensure actively growing (trophic) amoebae were always used. Moreover, a small block size (2 mm × 2 mm) containing approximately 50 amoebae was used to initiate the experimental plates by placing the block face containing the amoebae directly onto the start of the *E. coli* streak.. This ensured that amoebae could readily migrate from below the block and rapidly realize the experimental conditions, namely an aerobic or microaerophilic environmment. For practical purposes, it was not possible to measure actual growth rate. Rather, the distance migrated from the point of inoculation within a 5 day experimental treatment was recorded in mm. It is important to note that migration was a function of cell division since individual cells did not migrate a detectable distance. Cells migrated, and reproduced, along an *E. coli* streak as shown in Fig. 2. Here, the advancing front (arrow) consisting of hundreds of amoebae had migrated 40 mm from the site of inoculation.

To further underscore the reproductive potential along the bacterial streak, the number of cells of strain FSP2M was counted after 5 d incubation under microaerophilic conditions. This was the strain that migrated furthest out of all the acanthamoebae tested. Cells (ca. 50) were inoculated onto three replicate plates with *E. coli* streaks. After incubation amoebae (trophs and cysts) were fixed in 5% gluteraldeyde in amoeba saline and all cells were removed from the plates using a cell scraper and water rinses from a Pasteur pipette. Volumes were adjusted to 15 ml and counts were made using a Sedgwick Rafter counting chamber (× 100 magnification; address). For each plate, 20 replicate counts were performed (i.e. 20 × 1 µl).

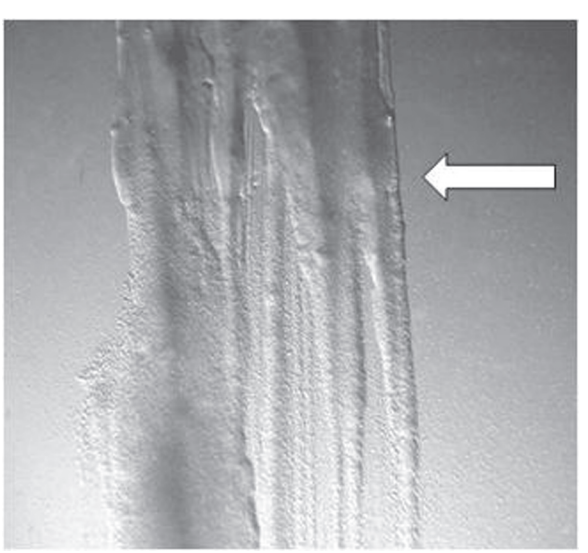

Fig. 2.

RESULTS

Thirteen strains of *Acanthamoeba* were subjected to culture under aerobic and microaerophilic conditions (approx. 11% air saturation). All strains grew under both conditions as evidenced by their migration along an *E. coli* streak on the NNA surface. Distance traveled was a function of growth since the advancing front was comprised of exponentially growing acanthamoebae. To emphasize the growth potential of acanthamoebae cultured on an agar plate, the number of cells was counted in the case of strain FSP2M under microaerophilic conditions. Here, plates contained a mean of 1.13 +/– 0.18 (S.D.) × 10^6 cells after 5 days incubation indicating that the population had doubled approximately 14 times. This implies that the generation time was of the order of 8.6 h. There were significant differences between the different strains of acanthamoebae with strain DS migrating (growing) least and strain FSP2M advancing the most (Fig 3).

However, the salient comparisons were between air and microaerophilic cultures and surprisingly eleven of the strains (85%) migrated significantly further (and presumably grew faster) under low oxygen conditions ($p > 0.05$; t-test; $n = 15$). All of these strains were genotype T4 and included the four known pathogenic strains tested (strains FLA1, 2, and 9; MS26; DS; P1,6 91 and 209; and FSP1M and FSP2M). This genotype is the one most frequently isolated from AK patients. Strain DS was also genotype T4, identical in sequence to the type-strain of the species, *A. castellanii* (Booton *et al*. 2004). Some authors consider this to be the main genotype involved in AK infections (Wang and Ahearn 1997). The distances migrated by the T3 and T5 isolates (P120 and TIV, respectively) tested in air and under low oxygen were not statistically different.

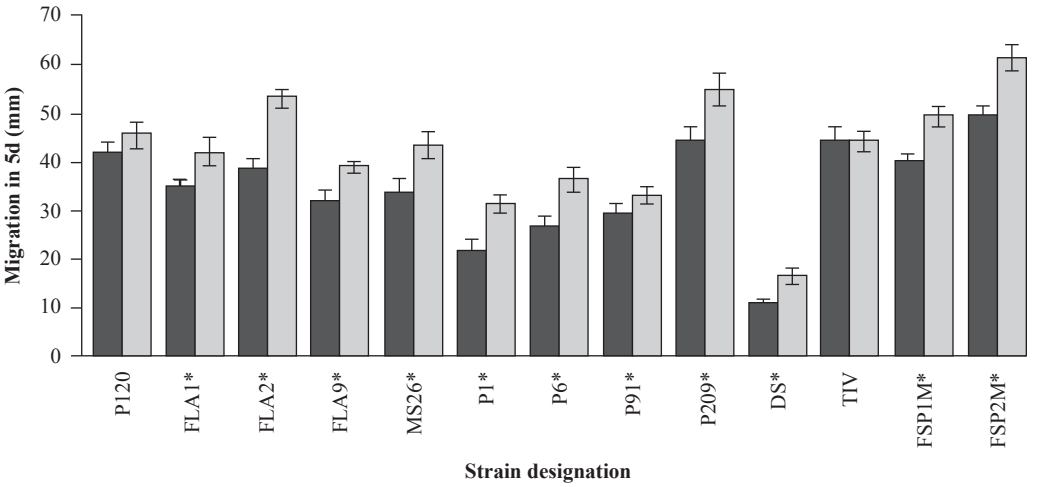

Fig. 3. Distance migrated on an agar plate (mm) after 5d of *Acanthamoeba* strains grown aerobically (shown in black) and microaerophilically (shown in white). All strains tested were T4 genotype except strain P120 (T3) and TIV (T5). Asterisks denote statistically significant differences (t-test: two sample assuming equal variances; $p < 0.05$).

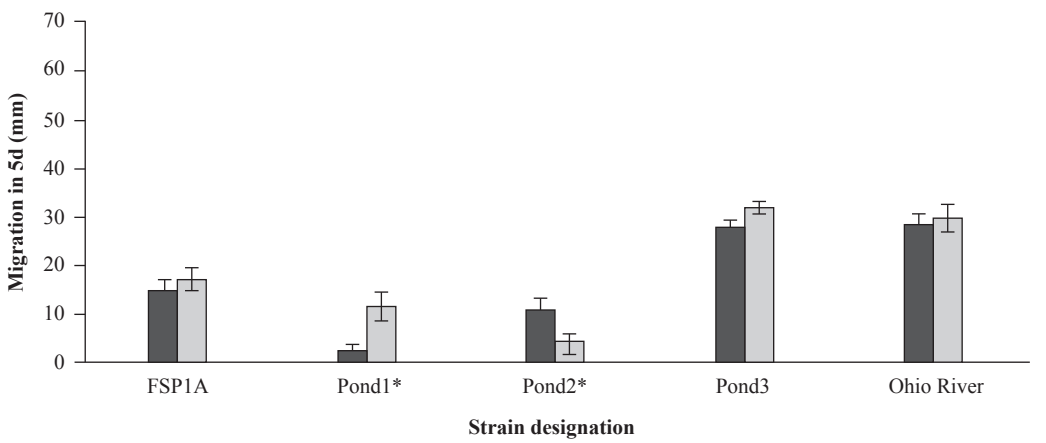

Fig. 4. Distance migrated on an agar plate (mm) after 5d of free-living amoebae (non- acanthamoebae) grown aerobically (shown in black) and microaerophilically (shown in white). Asterisks denote statistically significant differences (t-test: two sample assuming equal variances; $p < 0.05$). Strains indentifications: FSP1A (unidentified vahlkampfiid sp. 1), Pond 1 (*Vannella*), Pond 2 (unidentified thecate-like amoeba), Pond 3 (*Saccamoeba*), Ohio River (unidentified vahlkampfiid sp. 2).

These results for acanthamoebae were largely unexpected and to test whether improved growth for T4 acathamoebae grown under reduced oxygen was specific to these strains, or a generalized phenomenon for naked amoebae in general, the migration of five non-acanthamoebae strains were compared. Only strains that grew under the same conditions (those isolated on NNA with *E. coli*) were used. The data are shown in Fig. 4 and it is clear that most amoebae did not migrate as far as acanthamoebae, suggesting slower growth rates, although it is noteworthy that all species grew in both aerobic and microaerophilic conditions. The two vahlkampfiids (probably different genera) and *Saccamoeba* all showed equivalent migration under both conditions. However, the *Vannella* migrated significantly faster under low oxygen while the thecate amoeba did significantly better in air.

DISCUSSION

Bacterial biofilms are viewed as important sites of biotic interaction with an estimated 99% of microbial activity in freshwater existing within surface-associated communities (Bryers 1982). In a comprehensive review of protozoan grazing in freshwater biofilms, Parry (2004) emphasized the lack of studies on protozoa within biofilms, particularly in the case of amoebae that feed directly on biofilm-associated prey. While it is unclear whether the biofilm matrix is a source of nutrition for some protozoa (Parry 2004), it has been shown that the exopolymer matrix exuded by bacteria does not afford protection from protozoan grazing (Heaton *et al.* 2001). Likewise, the belief that alginate production by biofilm bacteria such as *Pseudomonas aeruginosa* and the formation of microcolonies (too large to ingest) offer protection from predation by amoebae has been discounted (Weitere *et al.* 2005). Whole genome sequencing of *A. castellanii* revealed the presence of a gene for alginate lyase. In that study, it is suggested the presence of this gene may account for the ability of *A. castellanii* to break down biofilms and thus feed on the biofilm bacteria (Anderson *et al.* 2005). It is also relevant to note that some amoebae can digest complex carbohydrates such as those found in the resistant spores of fungi and the phycolloid wall of a range of seaweeds (Old 1977, Polne-Fuller *et al*. 1990) presumably through the release of localized extracellular enzymes. It follows that amoebae can most likely migrate to the deepest parts of a biofilm where oxygen levels are reduced because of high bacterial metabolic rates.

Studies to elucidate the distribution of oxygen in biofilms have relied on assumptions about the chemical and biotic microenvironment within a film that are seldom, if ever, verified. However, Kuhl *et al.* (2007) recently used novel coverslip oxygen sensors to image the distribution of oxygen in biofilms. Their study showed the presence of pronounced O_2 gradients and anoxic microniches in the biofilm at a scale of 10 to 40 μm. Such quantification is not unexpected since microbial ecologists have assumed the metabolic activity of aerobic bacteria would reduce oxygen levels in a film. Thus, in the natural environment, soil amoebae will encounter microaerobic and anaerobic conditions from time to time (Turner *et al.* 1997). Similarly, within water treatment plants where biofilms are of paramount importance and can be very evident (ranging in thickness from 0.07 to 4.0 mm), inadequate oxygen in an aerobic wastewater treatment plant may be a cause of process failure. Indeed, a minimum dissolved oxygen level of 2 mg/L (ca. 26% water saturation) is commonly accepted as a requirement for proper performance of aerobic systems (Vesilind 2003).

Given the possible importance of amoebae grazing tightly associated bacteria deep within a biofilm by virtue of their predatory behavior and possible potent enzymes it follows that their tolerance to low oxygen should be considered. As noted in the introduction there have been several early studies considering the fate of amoebae facing anaerobic conditions (see Kitching 1939) but none addressing whether amoebae might be adapted to favor low oxygen environments. It should be noted here that acanthamoebae grown under anaerobic conditions failed to grow and were slow to encyst (authors SS and WT, unpublished). The present study used distance migrated (mm), a presumed index of growth, to compare the behavior of several strains of *Acanthamoeba* and species of amoeba to microaerophilic conditions (11% air saturation) and aerobic conditions. Migration of cells is a convenient comparative index for amoebae since they only migrate from the site of inoculation as a function of growth. For example, in the case of acanthamoebae, the 50 cells inoculated on day one generated a population of 1.13×10^6 cells after 5 days. All amoebae examined in the present study grew well under both aerobic and microaerophilic conditions. Of the 18 strains or species compared, 12 migrated significantly better under reduced oxygen supporting the view that biofilms can increase the concentration of free-living amoebae (Barbeau and Buhler 2001). Of the remaining 6 strains/species, only one isolate migrated significantly further under aerobic conditions. This was a thecate-like amoeba and the reasons for this result are unknown but nonetheless interesting. This may reflect a different niche preference for this species perhaps reflecting a habitat requirement in a fully oxygenated zone with different prey preferences. Certainly, some thecate amoebae have been shown to ingest microalgae and other protists (author AR unpublished). It is also interesting to note that *Vannella* sp markedly preferred microaerophilic conditions which supports observations in the laboratory where vannellid amoebae are easy to culture in dishes grazing on bacteria attached to the dish surface frequently below dense biofilms.

No previous studies have attempted to site low oxygen preference as a pathogenicity factor in the etiology of amoebic keratitis infections, however, such links have been made for bacterial pathogens. For example, biofilm

formation has been suggested to play a role in the environmental survival of the microaerophilic human pathogen *Campylobacter jejuni* (Reuter *et al.* 2010). Clearly, the survival and probable growth of *C. jejuni* under these conditions may help to produce an infective dose. While the number of *Acanthamoeba* needed to cause amoebic keratitis is unknown, it is likely that proliferation of cells on a lens prior to wearing is an important factor. As noted by Barbeau and Buhler (2001) who studied amoebae in dental unit waterlines, biofilm formation may favor the proliferation of amoebae. Some studies have also suggested that the ability of certain strains of acanthamoebae to tolerate harsh environmental conditions (such as elevated temperatures or high osmolarity) correlate with a predisposition towards pathogenicity (DeJonckheere 1983, Kahn *et al.* 2002).

The vast majority of amoebic keratitis infections are due to *Acanthamoeba*, although on rare occasion other genera have been isolated from the eye (*Hartmannella*, *Vahlkampfia* and *Naegleria*; Kennedy *et al.* 1995, Aitken *et al.* 1996, Dua *et al.* 1998). While it is pure speculation at this stage, perhaps any strain or species that can reproduce rapidly amid a biofilm on a lens surface pose a danger of becoming an infective dose. Most acanthamoebae infections are thought to be related to improper cleansing of lenses or storage cases (Stehr-Green *et al.* 1987) resulting in contamination by bacteria. If an amoebal cell is introduced either as a trophozoite or a cyst from the environment or, as some believe, from rinsing lenses with tap water containing amoebae (Shoff *et al.* 2007b), subsequent rapid multiplication of acanthamoebae could generate a sizeable population on the lens and ultimately the cornea of the eye. In short, the ability of an isolate to rapidly reproduce within a biofilm at reduced oxygen tensions may be an important factor in the establishment of an infective dose of amoebae.

There is some support for this notion in the data comparing all the strains of acanthamoebae. Of the 13 strains tested, 11 migrated, and presumably grew, significantly faster under low oxygen. All these strains were T4 genotypes, the most common type isolated from infected corneas, although these are also the most frequently encountered genotypes from environmental samples (Booton *et al*. 2004). Included in these 11 strains were four known pathogens from infected patients and the presumed pathogen *A. castellanii.* By contrast the two other genotypes tested (T3 and T5) did not migrate further under low oxygen conditions. Infections are not exclusively the domain of T4 genotypes and on rare occasions T3 and others have been isolated from the eye (Leedale *et al*. 1996). However, the data suggest that fast reproducing strains amid a biofilm and reduced oxygen may be a factor in the etiology of this disease. Certainly, the generation time estimated for the fastest migrating strain under low oxygen (strain FSP2M, 8.6 h) is short relative to published data. Heal (1967) compared the generation times of *Acanthamoeba* over a range of temperatures (5 to 25^{o}C) and found division times between 17 to 240 h.

It is now well known that the closed eye ocular tear film differs both chemically and physiologically when compared with the open eye ocular tear film (Sack *et al.* 1992, 2000, 2003). In fact, as Sack *et al.* (1992, 2000, 2003) have demonstrated, the closed eye corneal – ocular tear film interface represents a sub-clinical inflammatory milieu. The level of anti-bacterial lacrimal gland proteins decreases while complement and other inflammatory factors are activated. Oxygen levels are reduced from a partial pressure of 21% in the open eye to 8% in the closed eye (Liesgang 2002). The upregulation of an inflammatory cascade in the ocular tear film in association with corneal swelling leads to the loosening of corneal surface cell tight junctions in a way that would allow for the opportunistic penetration of the cornea by a pathogen such as *Acanthamoeba*. A number of investigators reported increased bacterial cell binding to rabbit and human corneal epithelial cells when exposed to hypoxic conditions (Imayasu *et al.* 1994, Solomon 1996, Fleiszig *et al.* 1992). Indeed, Fleiszig *et al*. (2010) demonstrated the formation of biofilms on the posterior surface of a contact lens where ambient oxygen levels would be lowest rather than the anterior contact lens surface. In our study, we have demonstrated that certain strains of *Acanthamoeba* migrate further (a consequence of growth) under microaerophilic conditions and more quickly attain a population density that may constitute an infective dose. The combination of decreased oxygen, increased microbial binding, upregulation of inflammatory factors in the ocular tear film and corneal surface cell morphological changes is a "perfect storm" of factors that will allow for the development of an infective outcome.

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