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Research Brief

Is Acanthamoeba pathogenicity associated with intracellular bacteria?

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A B S T R A C T

In addition to the possible role of Acanthamoeba as an evolutionary precursor of pathogenicity in microbial pathogens, it has been suggested that intracellular bacteria or other microbial endosymbionts may also enhance the pathogenicity of Acanthamoeba. Using transmission electron microscopy, polymerase chain reaction and simple culturing, our findings did not reveal any apparent evidence of microbial presence intracellularly of a recently recovered clinical isolate of Acanthamoeba. Based on these findings, it is tempting to speculate that the virulence of Acanthamoeba may not be attributed to the pathogenicity of the endosymbiont alone.

Several lines of evidence suggest that Acanthamoeba is a “Trojan horse” of the microbial world and can host viral, bacterial, fungal and protozoal organisms but who is the beneficiary of these interactions remains incompletely understood (reviewed in Khan, 2009). In addition to the possible role of Acanthamoeba as an evolutionary precursor of pathogenicity in microbial pathogens, it has been suggested that bacteria or other microbial endosymbionts may also enhance the pathogenicity of Acanthamoeba. Although, the results have been inconclusive, there are a few reports suggesting that bacterial endosymbionts enhance the virulence of Acanthamoeba (Badenoch et al., 1990; Fritsche et al., 1993; Fritsche et al., 1998; Xuan et al., 2007; Iovieno et al., 2010). For example, it has been shown that endosymbiont-infected Acanthamoeba produced a statistically significant enhancement in causing cytotoxic effects in comparison to uninfected Acanthamoeba, in vitro and in vivo (Badenoch et al., 1990; Fritsche et al., 1993; Fritsche et al., 1998). Based on these findings, it is hypothesized that the virulence of Acanthamoeba is attributed to the pathogenicity of the endosymbiont, which may be the real culprit in producing Acanthamoeba infections. The objectives of this study were to determine the presence of intracellular bacteria in a recently recovered Acanthamoeba isolate from a keratitis patient.

A clinical isolate of Acanthamoeba was cultured from an infected human cornea from a patient suffering with keratitis, presented at the Moorefields hospital, London, England, UK. Acanthamoeba isolate was grown axenically without shaking in PYG medium of proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v) in T-75 tissue culture flasks containing 10 ml of PYG medium at 30 °C. The medium was changed 18–24 h prior to any experimentation to ensure that the flask contained actively growing and feeding trophozoites (Borne, 1970; Khan and Siddiqui, 2009; Mortazavi et al., 2010). Based on morphological characteristics of cysts, it was considered as a Group 2 isolate. Using genus-specific primers, JDP1 and JDP2, the identity of Acanthamoeba was confirmed using the polymerase chain reaction as previously described (Booton et al., 2002). The pathogenicity of Acanthamoeba was confirmed in vitro by incubating 10⁶ amoebae/0.5 ml/well with human brain microvascular endothelial cells grown in 24-well plates and incubated overnight at 37 °C in a 5% CO₂ incubator. The next day, the supernatants were tested for LDH release using cytotoxicity detection assays as previously described (Alsam et al., 2005). Under these conditions, amoebae produced more than 60% cell death.

To determine the presence of cultivable intracellular bacteria in the clinical isolate, 10⁶ amoebae were incubated in 10 ml of LB, in T-75 tissue culture flasks and incubated for up to 96 h at 37 °C to observe the growth of bacteria that are possibly present intracellularly of Acanthamoeba as previously described (Alsam et al., 2005; Alsam et al., 2006). In some experiments, amoebae were disrupted using cell homogeniser and lysates incubated in 10 ml of LB as well as plated onto nutrient agar plates. Additionally, 10⁶ amoebae were incubated in 1% Triton-X-100, homogenized and lysates were incubated in 10 ml of LB, as well as plated onto nutrient agar plates. In some experiments, amoebae were lysed using 0.5% SDS
and lysates were incubated in 10 ml of LB as well as plated onto nutrient agar plates. All experiments were repeated three times using different passages of amoebae. Under these conditions, there was no evidence of cultivable bacteria.

In addition, polymerase chain reaction methods were performed using bacterial 16S rRNA universal primers (Bac-F 5'-AAC TGG AGG AAG GTG GGG AT-3' and Bac-R 5'-AGG AGG TGA TCC AAC CGC-3'). The DNA was extracted using routine proteinase K procedures and the PCR reaction performed at 95 °C for 5 min, cycled 35 times for 30 s each time at 95, 55 and 72 °C, and incubated for 10 min at 72 °C. *Escherichia coli* K-12 strain HB101 was used as a positive control. The PCR products were visualized using ethidium bromide on a 1% agarose gel, which proved negative for bacterial presence.

To observe pathogens intracellular of clinical isolate of *Acanthamoeba*, TEM was performed as previously described (Dudley et al., 2009). TEM is the gold standard technique to verify the intracellular localization of bacteria. Briefly, samples were fixed using 3% glutaraldehyde in a 0.2 M cacodylate buffer at 4 °C overnight. The next day, the samples were centrifuged as above and the pellet re-suspended in 0.1 M cacodylate buffer to wash any traces of glutaraldehyde. This process was repeated three times. The samples were post-fixed using 1% aqueous osmium tetroxide for 60 min at room temperature and dehydrated in a series of ethanol and water mixtures from 30% ethanol in water to 100% ethanol and finally using propylene oxide. The samples were embedded in Araldite epoxy resin and heated at 60 °C for 24 h. Ultrathin sections (approximately 0.01 μm thick) were cut with a with a diamond knife on a Reichert Ultracut E ultramicrotome and stained in 5% uranyl acetate for 5 min followed by Reynold’s lead citrate for 10 min. Stained thin sections were viewed under a Fei Tecnai BioTwin transmission electron microscope. In the results, there was no apparent evidence of microbial endosymbionts intracellularly of *Acanthamoeba* trophozoites and/or cysts, as shown in the representative TEM images (Fig. 1). The trophozoites showed the presence of a large number of mitochondria (Fig. 1A and B), while the cysts showed the characteristic double-wall (Fig. 1C and D).

In addition, TEM was used to observe various stages of *Acanthamoeba* encystment. Briefly, encystment was induced by inoculating amoebae onto non-nutrient agar and plates incubated at 30 °C.

![Fig. 1](image-url) Transmission electron microscopy revealed no obvious presence of microbial endosymbionts intracellular of *Acanthamoeba* trophozoites or cysts. To determine the presence of any microbial organisms within amoebae, various samples of trophozoites and cysts were observed under TEM. None of the samples tested showed any evidence of noticeable microbes intracellular of amoebae. Bar for A is = 5 μm; while bar for B, C, D is = 2 μm. The results are representative of three independent experiments and in each experiment 50 random sections of the slide were selected for observations.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Trophozoite</th>
<th>Early cyst</th>
<th>Mature cyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>26.2 μm</td>
<td>Becoming Round</td>
<td>16.2 μm</td>
</tr>
<tr>
<td>Shape</td>
<td>Irregular</td>
<td>Plasma membrane + amorphous outer layer</td>
<td>Spherical</td>
</tr>
<tr>
<td>Wall</td>
<td>Plasma membrane</td>
<td>Plasma membrane + endocyst + ectocyst</td>
<td>Survive harsh environments</td>
</tr>
<tr>
<td>Able to:</td>
<td>Feed, breed, move</td>
<td>Smaller groups with dark staining material</td>
<td>Small groups spread throughout</td>
</tr>
<tr>
<td>Golgi Bodies</td>
<td>Large groups</td>
<td>Coiled Structures seen</td>
<td>Smaller and more Condensed</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical analysis: (μg/million cells)</td>
<td>Dry weight: 706</td>
<td>Protein: 489</td>
<td>Glycogen: 83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1

Key differences at different stages of differentiation of *Acanthamoeba* (keratitis isolate belonging to the T4 genotype).
Jung et al., 2007). At various time intervals, amoebae were scraped off the agar surface. Amoebae were harvested by centrifugation at $3000 \times g$ for 10 min and processed for TEM as described above. This allows for changes in the morphology of the cell as well as any intracellular changes to be identified and monitored as the trophozoite undergoes differentiation. The results revealed morphological and intracellular changes within 6 h, which became significant within 12 h, as evidenced by the circular formation that the organelles have taken around the nucleus (Fig. 2C). There was an absence of any organelles outside this circular region, which became prominent with time (Fig. 2D). By 48 h, a double-walled cyst was observed within the trophozoite, with many intracellular organelles including mitochondria left out in the discarded region of the cell (Fig. 2E), that subsequently led to the formation of a complete cyst (Fig. 2H). Fig. 2G shows the presence of an ostiole, where the double-wall is absent and a thin membrane is used by the cyst to monitor environmental conditions. Under favorable conditions, the ostiole membrane is disintegrated and amoebae emerge as the vegetative trophozoite form. The key differences at various stages of Acanthamoeba differentiation are listed in Table 1.

In conclusion, these findings did not reveal any apparent presence of microbial endosymbionts intracellularly of the clinical
isolates of *Acanthamoeba*. Based on these findings, it is tempting to speculate that the virulence of *Acanthamoeba* may not be attributable to the pathogenicity of endosymbionts alone, however further studies using recently recovered keratitis isolates are needed to substantiate these findings. It is also envisaged that using eukaryotic organisms such as *Acanthamoeba* as a model organism, new mechanisms of how cells (e.g., stem cells) differentiate may hopefully be deduced. The use of *Acanthamoeba* as models is not uncommon, as they have been used to determine cellular mechanisms, for example the process of phagocytosis (reviewed in Khan, 2009). In addition, this unicellular organism has been used extensively to understand the molecular biology of motility. *Acanthamoeba* does not differ greatly at the ultrastructural-level from a mammalian cell, thus presents an excellent model for cell biology studies.

**References**


