Molecular Detection of Bacterial Endosymbionts in *Acanthamoeba* spp.: A Preliminary Study

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**ABSTRACT**

*Acanthamoeba* spp. are free-living amoeba that are commonly found in the environment. It is an agent of *Acanthamoeba* Keratitis (AK) and granuloma amoebic encephalitis (GAE). Amoeba can also serve as a host to various bacteria including those that are pathogenic such as *Mycobacterium*, *Legionella* and *Staphylococcus aureus* methicillin-resistant (MRSA). Based on this information, a study was conducted to detect the presence of three important medical bacteria endosymbionts in *Acanthamoeba* spp. that have been isolated from the cooling coils in the operating and delivery rooms in the Universiti Kebangsaan Malaysia Medical Centre. The presence of these endosymbiotic bacteria was screened using specific primer sets in conventional PCR and confirmed with sequencing analysis. Twenty-nine (80.56%) *Acanthamoeba* spp. samples were found to contain pathogenic bacteria endosymbionts within at least one bacterial genus per sample. *Mycobacterium* (82.76%) was the most commonly found, followed by *Legionella* spp. (65.52%) and *Pseudomonas* spp. (62.07%). No MRSA was detected from any sample in this study. Two endosymbiotic *Mycobacterium* that were identified have been grouped under *Mycobacterium tuberculosis*. We conclude that most *Acanthamoeba* have the potential to become hosts for various pathogenic bacteria, but the implications of this interaction on the pathogenicity of both organisms are still unclear and need further investigation.

Keywords: *Acanthamoeba*, bacteria endosymbiont, *Legionella*, MRSA, *Mycobacterium*, *Pseudomonas*
ABSTRACT

*Acanthamoeba* spp. is a free-living amoeba commonly found in the environment. It is the causative agent of *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). This amoeba is also a host to various bacteria including pathogenic ones such as *Mycobacterium*, *Legionella*, *Pseudomonas* and Methicillin-resistant *Staphylococcus aureus* (MRSA). In light of this information, a study was undertaken to detect these bacterial endosymbionts in *Acanthamoeba* spp. isolated from air-conditioning outlets in wards and operating theatres in Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The presence of these bacteria was screened using primer pair targeting each genus and further confirmed by sequencing analysis. Twenty-nine (80.56 %) *Acanthamoeba* isolates were found to contain targeted bacterial endosymbiont with at least one genus of bacteria per isolates. *Mycobacterium* spp. (82.76 %) were the most common bacteria detected, followed by *Legionella* spp. (65.52 %) and *Pseudomonas* spp. (62.07 %). No MRSA were detected in any isolates used in this study. Most of the *Mycobacterium* endosymbionts were non-tuberculous mycobacteria, while only two were part of *Mycobacterium tuberculosis complex* group. We conclude that most *Acanthamoeba* have the potential to host various pathogenic bacteria. However, the implication on the pathogenicity of both organisms remains unclear and further investigations are needed.

Keywords: *Acanthamoeba*, endosymbiont bacteria, *Legionella*, MRSA, *Mycobacterium*, *Pseudomonas*

INTRODUCTION

*Acanthamoeba* spp. is a free living amoeba which is found abundantly in both natural and man-made environment (Illingworth & Cook 1998; Chomicz et al. 2010). It is associated with life-threatening central nervous system infection in human (Khan 2006) and serves as reservoir for other microorganisms; indirectly provides protection from harsh elements in the environment. *Acanthamoeba* was also referred to as the ‘Trojan horses’ (Barker & Brown 1994) to viruses, bacteria, protists and yeast pathogens (Siddiqui & Khan 2012) and plays a major role in controlling microbial communities (Greub & Roult 2004).

Normally, ingested microorganisms are rapidly killed and digested by the host cell’s phagocytosis process and the same is seen in amoeba. However, several bacteria develop mechanism and survival strategies allowing them to resist phagocytosis pressure by *Acanthamoeba* (Molmeret et al. 2005; Akya et al. 2010). The capabilities of *Acanthamoeba* serving as a reservoir for bacteria received lot of interest (Greub & Raoult 2004) as most of these organisms were pathogenic to
humans.

Interaction of obligate intracellular bacteria with *Acanthamoeba* may involve either transient or stable association (Schmitz-Esser et al. 2008). *Legionella* and several bacterial species, such as *Mycobacterium avium*, *Chlamydia pneumoniae* and *Lysteria monocytogens*, were shown to survive and multiply in amoebae (Segal & Shuman 1999). The presence of these bacteria may enhance the virulence of *Acanthamoeba* (Cirillo et al. 1997).

It has been proposed that bacterial virulence may be a consequence of adaptations associated with intramoebal survival (Molmeret et al. 2005). Thus, this preliminary study was carried out to detect the presence of potential pathogenic bacterial endosymbionts in *Acanthamoeba* spp. isolated from air-conditioning vents in wards and operating theatres in UKMMC, Kuala Lumpur, Malaysia.

**MATERIAL AND METHODS**

**SAMPLES**

A total of 36 *Acanthamoeba* isolates maintained at the Culture Laboratory, Department of Parasitology and Medical Entomology, UKMMC were used in this study. These isolates were cultured from dust samples in air-conditioning vents in operating theatres and wards from UKMMC. All isolates were maintained on non-nutrient agar and strictly followed the aseptic technique practiced in the laboratory.

**DNA ISOLATION, GENOTYPING AND SEQUENCING**

Amoebae were harvested to achieve the confluence according to Hooshyar et al. (2013). Extraction of genomic DNA was utilized using Genomic DNA Isolation Kit, Norgen Biotek, Canada. The DNA products were then stored at -20°C. Conventional PCR was performed using particular specific primer for each bacterium (Table 1) after optimization was achieved. Amplification of 16S rDNA of *Meca* genes were used for MRSA (Sakoulas et al. 2001), 16S-23S internal transcribed spacer (ITS) for *Mycobacterium* spp. and 23S-5S intergenic spacer (IGS) which target variable regions for both *Legionella* spp. and *Pseudomonas* spp. (Iovieno et al. 2011).

For amplification of a single target DNA bacterial endosymbiont sequence, template DNA was initially denatured at 95°C for 5 mins, followed by a total of 35 cycles at 94°C, 55-62°C (Table 1), and 72°C each for 30 seconds. The purified DNA culture from MRSA strain, *Legionella* and *Mycobacterium* from Amplirun DNA control (Vircell, Spain) were used as DNA template in positive control, included along with negative controls (without DNA template). The amplification products were visualized by 1.5% agarose gel electrophoresis and SYBR safe staining.

All positive amplicons for each bacterium were sent to GBST Bioscience and Technology Co., Ltd. (Malaysia) sequence laboratory for sequencing. A nucleotide blast (blastn) was done for DNA sequence analysis using local assignment search.
tool (Blast) and compared to those in GenBank (www.ncbi.nlm.nih.gov).

RESULTS

Twenty-nine out of 36 (80.56%) Acanthamoeba isolates examined yielded targeted endosymbionts except for MRSA (Table 2). *Mycobacterium* endosymbionts were found in 24 (82.76%) Acanthamoeba isolates. Sequencing analysis showed 22 *Mycobacterium* endosymbionts were clustered into Non-Tuberculosis *Mycobacterium* (NTM) group and identified as *Mycobacterium fortuitum*, *M. massiliense*, *M. abscessus*, *M. vanbaalenii*, *M. senegalense*, *M. trivial* and *M. vaccae*. One Acanthamoeba endosymbiont from the operating theatre (AC63) was found to contain DNA sequences with 99-100% similarity to both *M. tuberculosis* and *M. bovis* as well as for NTM. *Legionella* endosymbionts were found in 19 (65.52%) Acanthamoeba isolates. These endosymbionts had the highest sequence similarities to the partial sequences of *Legionella longbeachae*, *L. wadwaorthii*, *L. monrovica*, *L. massiliensis* and *L. feeleii* (between 97-100%).

*Pseudomonas* species were documented in 18 (62.07%) Acanthamoeba isolates. Sequences derived from this study showed that *Pseudomonas* endosymbionts were most similar to *Pseudomonas stutzeri*, *P. aeruginosa*, *P. denitrificans*, *P. chlororaphis* and *P. knackmussi* with percentage similarities of 95-100%.

DISCUSSION

The presence of a variety of endosymbiont bacteria in *Acanthamoeba* hosts have been
reported worldwide. However, the study of such relationship was not ascertained in Malaysia. In the present study, we examined 36 Acanthamoeba isolates obtained from dust samples collected from air-conditioning vents in ward and operating theatres at UKMMC for the presence of these medically important bacterial agents: Legionella, Pseudomonas, Mycobacterium and MRSA. Out of 36 isolates examined, 29 were found to possess at least one bacterium as an endosymbiont. The predominant endosymbiont belonged to the genus Mycobacteria (82.76%). The presence of internalized Mycobacteria is expected since these bacteria are able to inhabit a diverse range of natural environments (Adekambi et al. 2006). The relationship between Mycobacterium and Acanthamoeba has been described in literature and it is speculated that Acanthamoeba may contribute to the transmission and pathogenesis of diseases caused by Mycobacterium (both tuberculosis complex and non tuberculosis Mycobacterium). It has been shown that Acanthamoeba can protect Mycobacteria from chlorine and antibiotics and enhance its virulence (Cirillo et al. 1997; Mba Medie et al. 2011). The association of Acanthamoeba as host for Mycobacterium has also been reported by Thomas et al. (2006). Their study reported that the amoebal coculture technique may be a significant device for recovering new particular mycobacterial organism. The two endosymbiotic Mycobacterium spp. in this study were found to be genetically related to M. tuberculosis and M. bovis, an important causative agent of tuberculosis in human (Reed et al. 2006). Even though the ability of these Mycobacterium tuberculosis complex (MTC) organisms to be released from its amoebae host and infect humans is unclear (Mba Medie et al. 2011), the possibility of it becoming an environmental source of transmissions should not be taken lightly. The presence of these potentially pathogenic Mycobacterium hiding in the highly resistant Acanthamoeba cysts in air-conditioning vents underline the importance of considering this amoeba during sterilization and disinfecting process of space and equipment used in wards and operating theatres.

Legionella has been previously reported in water system including hospital building (Yu et al. 2007; Massoni et al. 2013). Acanthamoeba spp. was reported to be abundant in air-cooling system and appeared as fundamental variable for the spread of Legionnaire's disease (Atlas 1999), where intracellular multiplication in Acanthamoeba is considered a prerequisite prior to an outbreak (Horn et al. 2001). Eventhough L. pneumophila was not detected in the isolates, the presence of non- pneumophila Legionella should not be taken lightly, since they may also cause legionellosis especially in immunosuppressed patients (Muder & Yu 2002).

Detection of Pseudomonas spp. may indicate that Acanthamoeba probably feed on Pseudomonas spp. that are widely distributed in environment. Pseudomonas spp. are highly
Adaptable bacteria that have the ability to colonize various environmental niches (José Maschio et al. 2015). Few of them evolved their ability and were able to survive predation by amoeba as supported in previous study by Siddiqui and Khan (2012) where *Pseudomonas aeruginosa* were reported to be naturally presence in *Acanthamoeba* and was proven in this study, as well. One of the reasons that led to this association is the presence of biofilm, where it plays a major role in the pathogenesis of *Acanthamoeba* keratitis as its provide attractive niches for both *Acanthamoeba* and bacteria especially in contaminated contact lens storage in keratitis cases (Khan 2006; José Maschio et al. 2015).

The interaction between *Acanthamoeba* and other microbial agents caused a great concern in the ability of this amoeba to indirectly transport a pathogen from the environment to humans including possible effects of their interactions on the virulence and pathogenicity of both organisms in human infection. *Acanthamoeba* spp. has been exhibited to create endosymbiotic association with the various waterborne microscopic organisms including *Mycobacterium, Legionella* and *Pseudomonas* bacteria (Yu et al. 2007; José Maschio et al. 2015). The interaction might be vital in both the development and survival of these opportunistic pathogens in clinical and environmental isolates and their capabilities to infect the humans.

Since this is only a preliminary study, more data are required for better understanding on the interaction and pathogenicity pattern in both *Acanthamoeba* spp. and its endosymbiotic bacteria. Future study should include staining and the use of higher microscopy screening approach such as Fluorescent in situ Hybridization (FISH) to determine the type and viability of the bacteria present in the amoebaee. This is to ascertain whether the intracellular bacteria are still viable and able to infect human after being released upon the demise of the amoeba host.

**CONCLUSION**

We presumed that most *Acanthamoeba* spp. can possibly have different pathogenic microorganisms. In any case, the suggestion on the pathogenicity of both living beings stays vague and further examinations are required.

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