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ORIGINAL ARTICLE

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

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ABSTRAK

Acanthamoeba spp. merupakan ameba hidup bebas yang biasa ditemui di persekitaran. Ia merupakan agen penyebab keratitis Acanthamoeba (AK) dan ensefalitis ameba bergranuloma (GAE). Ameba ini juga mampu menjadi perumah kepada pelbagai bakteria termasuklah yang bersifat patogenik seperti Mycobacterium, Legionella dan Staphylococcus aureus rintang metisilin (MRSA). Berdasarkan maklumat ini, satu kajian dijalankan untuk mengesan kehadiran tiga bakteria endosimbion berkepentingan perubatan di dalam Acanthamoeba spp. yang telah dipencilkan dari bolong penghawa dingin yang terdapat di wad and dewan bedah di Pusat Perubatan Universiti Kebangsaan Malaysia. Kehadiran bakteria endosimbion ini disaring menggunakan pasangan primer khusus bagi setiap genus menggunakan reaksi rantai polimerase (PCR) konvensional dan disahkan dengan analisis penjujukan. Dua puluh sembilan (80.56%) pencilan Acanthamoeba spp. didapati mengandungi bakteria endosimbion patogenik yang disasarkan dengan sekurang-kurangnya satu genus bakteria bagi setiap pencilan. Mycobacterium (82.76 %) adalah bakteria yang paling banyak dikesan, diikuti dengan Legionella sp. (65.52 %) dan Pseudomonas spp. (62.07 %). Tiada bakteria MRSA dikesan daripada mana-mana pencilan dalam kajian ini. Dua endosimbion Mycobacterium yang dikenalpasti telah dikelompokkan ke dalam strain Mycobacterium tuberculosis. Kami membuat kesimpulan bahawa, kebanyakan Acanthamoeba berpotensi untuk menjadi perumah bagi pelbagai bakteria patogenik, namun implikasi interaksi ini terhadap patogenisiti kedua-dua organisma masih kurang jelas dan memerlukan penyelidikan yang lebih lanjut.

Kata kunci: *Acanthamoeba, bakteria endosimbion, Legionella, MRSA, Mycobacterium, Pseudomonas*

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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 intra-amoebal 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

Table 1: Primers (5′ to 3′) used for gene sequence typing of each bacterial endosymbiont small sub unit (SSU) 16S rDNA with corresponding PCR parameters

Bacteria	Primer	Sequence	Annealing Temperature
MRSA	*mecA*1	CTCAGGTACTGCTATCCACC	53ºC
	mecA 2	CACTTGGTATATCTTCACC	
Mycobacterium spp.	Sp1	ACCTCCTTTCTAAGGAGCACC	61ºC
	Sp2	GATGCTCGCAACCACTATCCA	
Legionella spp.	23S	TGAAGCCCGTTGAAGACTAC	58ºC
Pseudomonas spp.	5S	GGAAGCCTCACACTATCAT	

Table 2: Frequency of occurrence of bacterial endosymbionts found in *Acanthamoeba* spp. isolates (n = 29)

Bacterial endosymbionts	Number of isolates (%)
MRSA	0 (0)
Mycobacterium spp.	24 (82.76)
Legionella spp.	19 (65.52)
Pseudomonas spp.	18 (62.07)

tool (Blast) and compared to those in GenBank (www.ncbi.nml.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* while another isolate (AC49) had DNA sequences with less than 90% similarity to both *M. tuberculosis* and M. *bovis* as well as for NTM.

Legionella endosymbionts were found in 19 (65.5%) *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 co-culture 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 endosymbiont 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 amoebae. 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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