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ORIGINAL PAPER

Interactions of *Pseudomonas aeruginosa* and *Corynebacterium* spp. with non-phagocytic brain microvascular endothelial cells and phagocytic *Acanthamoeba castellanii*

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Abstract Several lines of evidence suggest that *Acanthamoeba* interact with bacteria, which may aid in pathogenic bacterial transmission to susceptible hosts, and these interactions may have influenced evolution of bacterial pathogenicity. In this study, we tested if Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Corynebacterium* spp. can associate/invade and survive inside *Acanthamoeba castellanii* trophozoites and cysts, as well as non-phagocytic human brain microvascular endothelial cells. The results revealed that both *Corynebacterium* spp. and *P. aeruginosa* were able to associate as well as invade and/or taken up by the phagocytic *A. castellanii* trophozoite. In contrast, *P. aeruginosa* exhibited higher association as well as invasion of non-phagocytic HBMEC compared with *Corynebacterium* spp. Notably, *P. aeruginosa* remained viable during the encystment process and exhibited higher levels of recovery from mature cysts (74.54 bacteria per amoebae) compared with *Corynebacterium* spp. (2.69 bacteria per amoeba) ($P<0.05$). As *Acanthamoeba* cysts can be airborne, these findings suggest that *Acanthamoeba* is a potential vector in the transmission of *P. aeruginosa* to susceptible hosts. When bacterial-ridden amoebae were exposed to favourable (nutrient-rich) conditions, *A. castellanii* emerged as vegetative trophozoites and remained viable, and likewise viable *P. aeruginosa* were also observed but rarely any *Corynebacterium* spp. were observed. Correspondingly, *P. aeruginosa* but not *Corynebacterium* spp. exhibited higher cytotoxicity to non-phagocytic HBMEC, producing more than 75 % cell death in 24 h, compared to 20 % cell death observed with *Corynebacterium* spp. Additionally, it was observed that the bacterial conditioned medium had no negative effect on *A. castellanii* growth. Further characterization of amoebal and bacterial interactions will assist in identifying the role of *Acanthamoeba* in the transmission and evolution of pathogenic bacteria.

Keywords *Acanthamoeba* · Brain microvascular endothelial cells · Association · Invasion · Survival · Encystment · *Pseudomonas aeruginosa* · *Corynebacterium* spp.

Introduction

Acanthamoeba, a unicellular protist pathogen, is widely dispersed in the environment. (Siddiqui and Khan 2012; Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007). Being free-living, *Acanthamoeba* can withstand harsh environments. *Acanthamoeba* exists in two distinct forms: an active trophozoite form during which it reproduces and a dormant cyst form where it remains inactive with little metabolic activity, nonetheless remaining viable for years. Notably, *Acanthamoeba* harbours diverse microbial organisms including viruses, bacteria, yeast and protists, some of which are potential pathogens (Khan 2006). The precise nature of this symbiosis is unclear, but it is proposed that these interactions enable pathogenic microbes to endure hostile conditions, and this association may aid in their transmission to susceptible hosts in order to establish infection. For this reason, *Acanthamoeba*-bacteria interactions have gained substantial

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attention from the scientific and medical community, leading to speculations of employing novel anti-amoebic approaches in eradicating "superbugs" from the clinical setting. For example, *Acanthamoeba* has been found to co-occur with *Mycobacterium* spp. and other superbugs within the hospital environment (Ovrutsky et al. 2013; Siddiqui et al. 2013).

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium. It can result in lung infections in cystic fibrosis patients and may infect any part of the human body in immunosuppressed and hospitalized patients with cancer, transplantation and burns (Giamarellou 2000; Lyczak et al. 2000). *P. aeruginosa* can be found in a number of habitats, including soil, water, plant and animal surfaces, and decaying organic matter; however, the precise ecological niche of *P. aeruginosa* is not well understood (Ferguson et al. 2001). *Corynebacterium* spp. are Gram-positive bacteria and are widely distributed in nature mostly being innocuous (Collins et al. 2004). However, some are known to cause human disease. *Corynebacterium diphtheriae*, for example, is the pathogen responsible for diphtheria. Being co-inhabitants of soil, it is likely that both *P. aeruginosa* and *Corynebacterium* spp. interact with *Acanthamoeba* in the environment. In this regard, amoeba has been suggested as the "training ground" during evolution of bacterial pathogenicity (Molmeret et al. 2005). Thus, it is important to understand interactions of pathogenic bacteria with *Acanthamoeba*. Here, we studied *P. aeruginosa* and *Corynebacterium* spp. interactions with the environmental phagocyte, *Acanthamoeba*, while non-phagocytic brain microvascular endothelial cells (BMEC) were used as a control. The human BMEC used in this study are primary in nature as well as non-phagocytic offering a useful model for comparative study. Additionally, it was determined whether these bacteria can survive intracellular of *Acanthamoeba castellanii* cysts, a property that can allow bacterial viability in the environment in the face of harsh conditions and allow their transmission to the susceptible hosts.

Materials and methods

Cultures of *A. castellanii*

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated. A clinical isolate of *A. castellanii* belonging to the T4 genotype, isolated from a keratitis patient (American Type Culture Collection, ATCC 50492), was used in the present study. Amoebae were grown without shaking in 15 mL of PYG medium [0.75 % (*w*/*v*) proteose peptone, 0.75 % (*w*/*v*) yeast extract and 1.5 % (*w*/*v*) glucose] in T-75 tissue culture flasks at 30 °C as previously described (Sissons et al. 2005). To obtain vegetative trophozoites, the media were refreshed 17–20 h prior to experimentation, which resulted in more than 95 % amoebae in the trophozoite forms.

Bacterial cultures and growth conditions

P. aeruginosa and *Corynebacterium* spp. were used in this study. *P. aeruginosa* is a clinical isolate from the Aga Khan University hospital, and *Corynebacterium* spp. are an environmental isolate as described previously (Siddiqui et al. 2013). All bacteria were grown aerobically in Luria-Bertani (LB) broth at 37 °C.

Human brain microvascular endothelial cell cultures

The primary human brain microvascular endothelial cells (HBMEC) were isolated from seizure patient who had undergone cerebral cortex resection as previously described (Stins et al. 1997). The HBMECs were grown in T-75 tissue culture flasks in RPMI-1640 containing 10 % heat-inactivated fetal bovine serum, 10 % Nu-serum, 2 mM glutamine, 1 mM Na-pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, non-essential amino acids and vitamins as previously described (Alsam et al. 2003; Stins et al. 1997).

Cytotoxicity assays

For cytotoxicity assays, 5×10^5 HBMEC per well per 0.5 mL were cultured in 24-well plates and incubated at 37 °C in a 5 % CO_2 incubator. Under these conditions, HBMEC formed complete monolayers within 48 h. *P. aeruginosa* and *Corynebacterium* spp. were grown in LB for overnight, and the optical density was adjusted to 0.22 absorbance at wavelength of 595 nm [equivalent to approximately 10^8 colony-forming units (c.f.u.)/mL]. Next, 10 μL containing 10^6 c.f.u. were transferred to 490 μL RPMI 1640 and inoculated in each well of a 24-well plate containing HBMEC monolayers. After 60 min of incubation, the monolayers were washed with PBS and incubated with gentamicin (100 μg/mL in RPMI-1640) for 60 min to kill extracellular bacteria. The wells were then washed twice with RPMI-1640, and plates were incubated at 37 °C in a 5 % CO_2 incubator and monitored for monolayer disruptions over the period of 24 h. Next, the supernatants were collected and centrifuged at 13,000×*g* for 5 min to remove cellular debris. Cytotoxic effects were determined by estimating the release of cytosolic lactate dehydrogenase in the medium (Cytotoxicity Detection kit; Roche Applied Sciences). The percent cytotoxicity was calculated as follows: % cytotoxicity = (sample value−control value) / (total LDH release−control value)×100. Control values were obtained by incubating HBMEC monolayers with 500 μL RPMI-1640 alone, and total LDH was released from HBMEC by completely lysing them in 500 μL 1 % Triton X-100. The LDH release from Triton X-100-treated HBMEC was

considered 100 % cell death, and bacterial-mediated cell death is expressed as the relative change.

Association assays

Assays were performed to determine if the bacterial strains associate with *A. castellanii* and HBMEC. For *A. castellanii*, amoebae were maintained in the trophozoite stage in tissue culture flasks in PYG medium. Upon confluency, the unbound amoebae were aspirated, and growing trophozoites were rinsed with phosphate buffer saline (PBS) pH 7.4. Next, 10 mL of PBS was added to the flask, and trophozoites were chilled on ice for 20 min and pelleted by centrifugation at 1,000*g* for 5 min. The cell pellet was resuspended in 1 mL of PBS, and the number of amoebae was counted using a hemocytometer (10^6 cells). For bacteria, the optical density of cultures was adjusted to 0.22 at 595 nm [equivalent to approximately 10^8 colony-forming units (c.f.u.)/mL]. Bacteria (10^7 c.f.u) were incubated with *A. castellanii* (10^6 cells) at 30 °C for 1 h for association assays as previously described (Yousuf et al. 2013). Briefly, following 1 h of incubation, co-cultures of amoebae plus bacteria were centrifuged at 2,000*g* for 2 min to limit non-associated bacterial pelleting. The supernatants were collected and plated on nutrient agar plats to determine the presence of non-associated bacteria. In contrast, the pellet was resuspended in 0.5 mL of PBS and vortexed by a brief pulse. This process was repeated three times. At the final wash, the discarded supernatant was also plated onto nutrient agar plates to determine bacterial presence. Notably, the first two washes revealed bacterial presence; however, the final wash did not reveal any bacterial presence. The amoebae were counted using a hemocytometer and then lysed by adding 0.5 % SDS for 10 min (this concentration lysed amoebae trophozoites but did not affect the viability of amoeba cysts or the bacteria tested in this study). The lysates containing bacteria were serially diluted and plated on nutrient agar plates and colonies enumerated the next day. The bacterial colony-forming units associated with *A. castellanii* were calculated as follows: recovered bacterial c.f.u. / number of *A. castellanii* = bacterial c.f.u./*A. castellanii* ratio.

To study bacterial interactions with human cells, HBMEC were grown to confluent monolayers in 24-well plates. Next, bacteria were incubated with HBMECs (10^7 c.f.u. per well in 0.5 mL) as described above and plates incubated at 37 °C in a 5 % CO_2 incubator. After 60 min of incubation, the monolayers were washed with PBS, and HBMEC were lysed by adding 500 μL of distilled water for 30 min together with gentle scraping of the well. The lysates were serially diluted and plated on nutrient agar plates and the colonies enumerated the next day.

Invasion assays

Assays were performed to determine if the bacterial strains are taken up by *A. castellanii* and HBMEC. Briefly, amoebae were incubated with bacterial strains as described for association assays. After washing with PBS thrice, the extracellular bacteria were killed by adding gentamicin (100 μg/mL in PBS for 90 min at 37 °C in a 5 % CO_2 incubator). Finally, amoebae and bacteria were enumerated as described above. The bacterial colony-forming units invading *A. castellanii* were calculated as follows: recovered bacterial c.f.u. / number of *A. castellanii* = bacterial c.f.u./*A. castellanii* ratio. For HBMEC, bacteria were incubated with HBMEC. After 60 min of incubation, the monolayers were washed with PBS and incubated with gentamicin (100 μg/mL in RPMI-1640) for 60 min to kill extracellular bacteria. The wells were then washed twice with RPMI-1640, and HBMEC lysed and bacterial c.f.u. were determined as described above.

Intracellular cysts survival assays

Intracellular cyst survival assays were performed to evaluate the ability of bacterial strains to survive inside *A. castellanii* cysts as described previously (Yousuf et al. 2013). In brief, following invasion assays, the mixtures were transferred onto non-nutrient agar plates [prepared using 3 % (*w*/*v*) purified agar]. The plates were incubated at 30 °C for up to 10 days. This allowed complete encystment of *A. castellanii* trophozoites into the cyst form, as observed visually under a phase-contrast microscope. Cysts were then gently scraped off the agar surface using a cell scraper by adding 5 mL of dH_2O and collected by centrifugation at 2,000*g* for 10 min and resuspended in 0.5 mL of PBS and counted using a hemocytometer. The cysts were treated with SDS (0.5 % final concentration), and the bacterial colony-forming units were determined by plating on nutrient agar plates. The bacterial colony-forming units surviving intracellular of *A. castellanii* cysts were calculated as follows: recovered bacterial c.f.u. / number of *A. castellanii* = bacterial c.f.u./*A. castellanii* ratio.

Intracellular HBMEC survival assays

Assays were performed to determine if the bacterial strains survive intracellular of HBMEC. Briefly, following invasion assays, the monolayers were washed twice with RPMI-1640, and HBMEC were incubated in 0.5 mL of RPMI-1640 for 24 h at 37 °C in a 5 % CO_2 incubator. Next, HBMEC were lysed by adding 500 of distilled water for 30 min together with gentle scraping of the well. The lysates were serially diluted and plated on nutrient agar plates, and the colonies enumerated the next day and bacterial c.f.u. were determined as described above.

Effect of favourable (nutrient-rich) and unfavourable conditions (nutrient-deprivation) on excystation of *A. castellanii* and bacterial growth

Following invasion assays, *A. castellanii* plus intracellular bacterial mixtures were transferred onto non-nutrient agar plates (prepared using 3 % (*w*/*v*) purified agar). The plates were incubated at room temperature for up to 10 days. This allowed complete encystment of *A. castellanii* trophozoites into the cyst form; this was confirmed visually under a phase-contrast microscope. Cysts were then gently scraped off the agar surface using a cell scraper by adding 5 mL of dH_2O and collected by centrifugation at 2,000*g* for 10 min and resuspended in 0.5 mL of PBS and counted using a hemocytometer. The scraped cysts were inoculated in different conditions: including PYG medium, LB medium, PBS and dH_2O and incubated at 30 °C for 72 h and observed under a phase-contrast microscope for effects on growth of both *A. castellanii* and bacteria.

Effect of *P. aeruginosa* and *Corynebacterium* spp. conditioned medium on the viability of *A. castellanii*

Assays were performed to evaluate the effect of bacterial conditioned medium on *A. castellanii* trophozoites. Bacterial conditioned media (CM) was prepared by inoculating *P. aeruginosa* and *Corynebacterium* spp. in RPMI-1640 medium overnight. The cell-free supernatant (i.e., CM) was collected by centrifugation at 13,000×*g* for 5 min and incubated with *A. castellanii* trophozoites (10^6 cells) at 30 °C for 2 h and 24 h. Following this, the mixtures were transferred onto plates containing PYG medium and growth were observed visually under a phase-contrast microscope at 24 h. For controls, *A. castellanii* (10^6 cells) were incubated with RPMI-1640 medium alone for 2 and 24 h and then incubated in PYG.

Results

Bacterial association with *A. castellanii* and HBMEC

To determine whether *P. aeruginosa* and *Corynebacterium* spp. associate with *A. castellanii* and HBMEC, association assays were performed. The findings revealed that both *P. aeruginosa* and *Corynebacterium* spp. exhibited association with *A. castellanii* (9.27 bacteria per amoeba ratio for *P. aeruginosa* and 45.3 bacteria per amoeba ratio for *Corynebacterium* spp. respectively (Fig. 1a), albeit *Corynebacterium* spp. exhibited higher association compared with *P. aeruginosa* ($P<0.05$, using two-sample *t* test; two-tailed distribution). Here, the term association represents bacteria both inside amoebae and those that were attached on the surface of *A. castellanii*. In contrast, *P. aeruginosa* exhibited higher association with non-phagocytic HBMEC compared with *Corynebacterium* spp. ($P<0.05$, using two-sample *t* test; two-tailed distribution) (Fig. 1b).

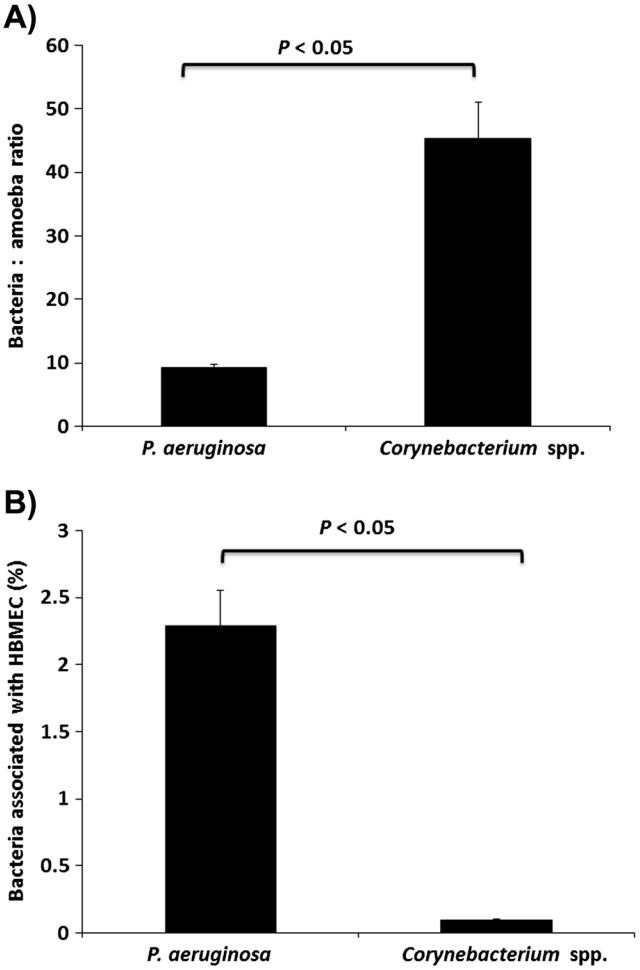

Fig. 1 *P. aeruginosa* and *Corynebacterium* were incubated with *Acanthamoeba* and HBMEC for 1 h. The unbound bacteria were removed by washing and associated bacteria enumerated as described in "Materials and Methods". **a** represents ratio of bacteria per amoeba, while **b** represents the number of bacteria associated with HBMEC (percent of the original inoculum). The results are the mean of three independent experiments performed in duplicate. *Error bars* represent standard error

Bacterial invasion/uptake by *A. castellanii* and HBMEC

Next, to determine whether *P. aeruginosa* and *Corynebacterium* spp. invade/taken up by *A. castellanii* and HBMEC, invasion assays were performed and the intracellular bacteria were determined. The results showed that *P. aeruginosa* exhibited invasion/uptake by *A. castellanii* (3.55 bacteria/amoeba ratio) (Fig. 2a). Similarly, *Corynebacterium* spp. exhibited invasion/uptake by *A. castellanii* at levels similar to *P. aeruginosa* (3.39 bacteria/amoeba ratio) (Fig. 2a). Of note, the PBS post-gentamicin wash, plated onto nutrient agar plates, did not

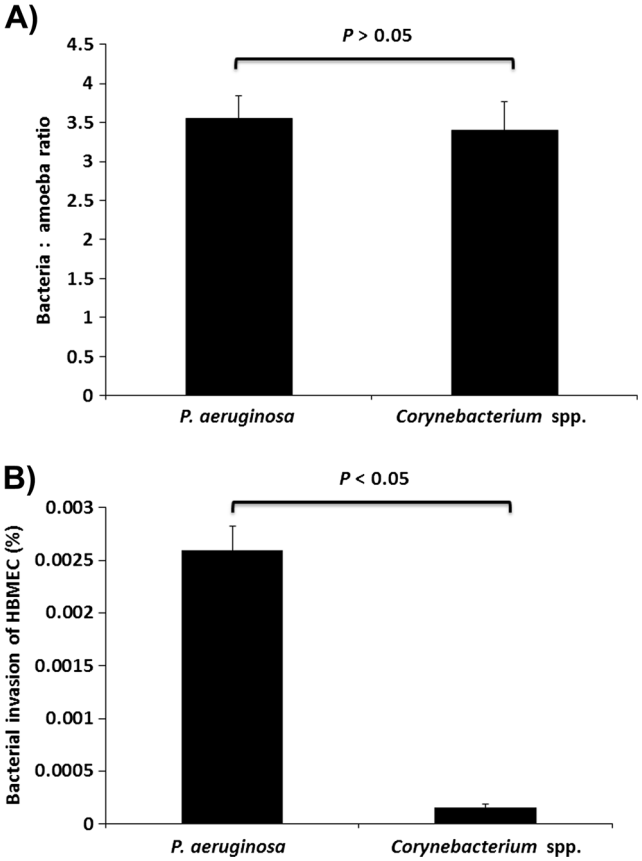

Fig. 2 *A. castellanii* and/or HBMEC were incubated with *P. aeruginosa* and *Corynebacterium* for 1 h, after which gentamicin was added to kill the extracellular bacteria, following which amoebae and intracellular bacteria were enumerated as described in "Materials and methods." **a** Represents ratio of bacteria per amoeba, while b represents the number of bacteria invading HBMEC (percent of the original inoculum). The results are the mean of three independent experiments performed in duplicate. *Error bars* represent standard error

yield any bacterial c.f.u, confirming that the antibiotic treatment was effective. In contrast, *P. aeruginosa* exhibited higher invasion of HBMEC compared with *Corynebacterium* spp. ($P<0.05$, using two-sample t test; two-tailed distribution) (Fig. 2b).

Bacterial survival of *A. castellanii* encystment process

The viability of *P. aeruginosa* and *Corynebacterium* spp., inside *A. castellanii* during encystment and recovery from mature cysts, was determined by performing intracellular cyst survival assays. The SDS treatment affected cyst viability (no growth observed in PYG) through lysing the ostiole membrane and leading to the recovery of any intracellular bacteria. The results revealed that both *P. aeruginosa* and *Corynebacterium* spp. remained viable during encystment, albeit *P. aeruginosa* exhibited significantly higher levels of recovery from mature cysts (74.54 bacteria per amoebae ratio) compared with *Corynebacterium* spp. (2.69 bacteria per amoeba ratio) ($P<0.05$, using two-sample t test; two-tailed distribution) (Fig. 3a).

For HBMEC, the results revealed that *P. aeruginosa* increased in number exponentially (Fig. 3b) and HBMEC monolayers were completely destroyed. In contrast, *Corynebacterium* spp. showed no damage to HBMEC as evident by intact monolayers and minimal bacterial recovery compared with *P. aeruginosa* ($P<0.05$, using two-sample t test; two-tailed distribution) (Fig. 3b). This was further confirmed using cytotoxicity assays, which revealed that *P. aeruginosa* exhibited significantly higher HBMEC cytotoxicity compared with *Corynebacterium* spp. ($P<0.05$) (Fig. 4).

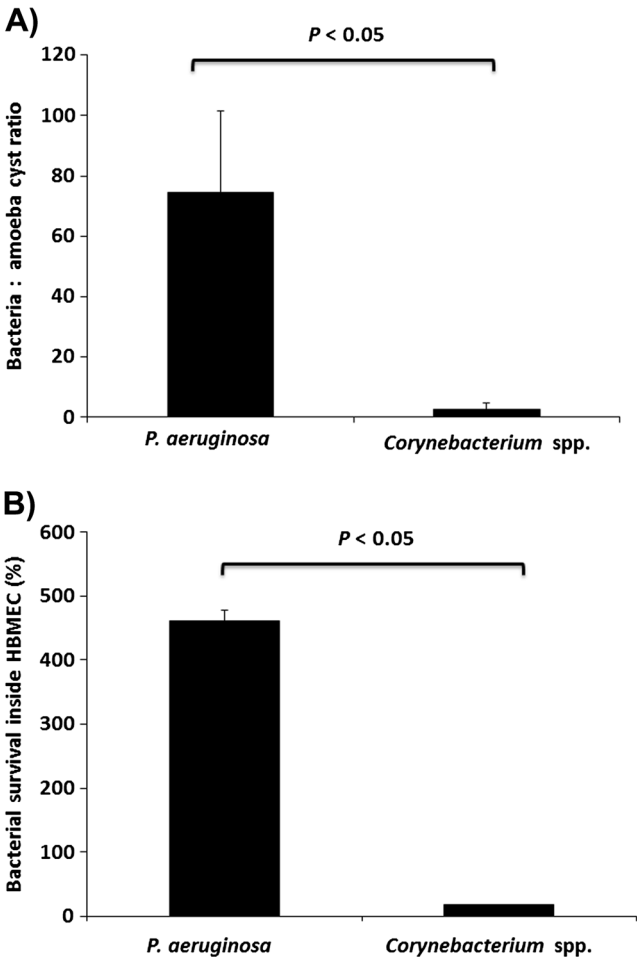

Fig. 3 **a** *A. castellanii* were incubated with each, *P. aeruginosa* and *Corynebacterium* for 1 h, followed by the addition of gentamicin to kill the extracellular bacteria. Finally, *A. castellanii* plus intracellular bacteria were inoculated onto non-nutrient agar plates for several days, followed by amoebae counting. The bacterial counts intracellular of cysts were calculated as described in "Materials and methods." The results are the mean of three independent experiments performed in duplicate. *Error bars* represent standard error. **b** HBMEC were incubated with each, *P. aeruginosa* and *Corynebacterium* for 1 h, followed by the addition of gentamicin to kill the extracellular bacteria and plates that were incubated for 24 h, followed by counting of intracellular bacteria as described in "Materials and methods." The results are presented as the mean ± standard error of three individual experiments performed in duplicate

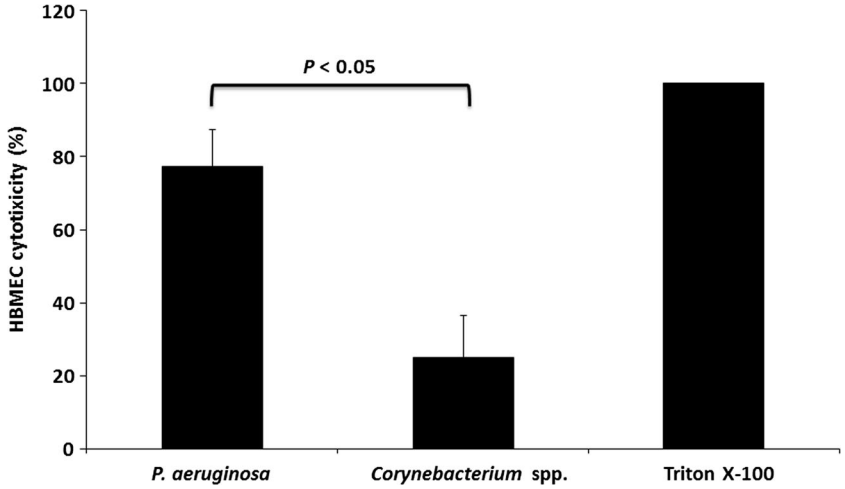

Fig. 4 HBMECs were incubated with each *P. aeruginosa* and *Corynebacterium* for 1 h, followed by gentamicin treatment as described in Fig. 2. Next, plates were incubated for 24 h at 37 °C in a 5 % CO_2 incubator, followed by the determination of cytotoxic effect of bacteria on HBMEC. The LDH release from 1 % Triton X-100-treated HBMEC was considered 100 % cell death, and bacterial-mediated cell death is expressed as the relative change. The results are the mean of three independent experiments performed in duplicate. *Error bars* represent standard error

A. castellanii and *P. aeruginosa* exhibited growth when bacterial-ridden amoebae cysts were exposed to favourable conditions (nutrient-rich)

Following survival assays, the mature cysts containing intracellular bacteria were inoculated in different conditions: PYG growth medium, LB growth medium, PBS and dH_2O. The results revealed that in all the nutrient conditions tested, *A. castellanii* trophozoites emerged and remained viable, when observed for up to 72 h, along with fewer numbers of *P. aeruginosa*, but *Corynebacterium* spp. were rarely observed (Table 1). For either PYG or LB, *A. castellanii* trophozoites emerged from bacterial-ridden cysts along with few *P. aeruginosa* were observed. However, when incubated with PBS or dH_2O; *A. castellanii* remained in the cyst form (Table 1). Of note, at all nutrient conditions tested, *P. aeruginosa* were unable to lyse or overgrow *A. castellanii* and vice versa.

Table 1 Bacterial-ridden mature amoebae cysts were inoculated in various conditions, and growth of *A. castellanii* and bacteria was observed after 72 h

Inoculation medium	*A.castellanii*	*Corynebacterium* spp.	*P.aeruginosa*
PBS	–	–	–
dH_2O	–	–	–
PYG	+++	+	+++
LB	+++	+	+++

Results are representative of several experiments

+++ is positive growth, + is rarely observed, – is no growth

Bacterial conditioned medium did not affect *A. castellanii* growth

Assays were performed to determine the effect of bacterial conditioned medium on *A. castellanii* growth. Bacterial conditioned media (CM) was inoculated with *A. castellanii* trophozoites. Following this, the mixtures were transferred to PYG medium, and growth was observed visually under a phase-contrast microscope for 24 h. The findings revealed that the presence of CM did not affect *A. castellanii* growth as viable amoebae were observed at all-time points tested and at numbers approximately similar to PYG alone.

Discussion

Acanthamoeba acts as a host for a variety of microbes, including viruses, bacteria, protists and yeast/fungi (reviewed in Khan 2009; La Scola et al. 2001, 2003). For this reason, *Acanthamoeba* are receiving profuse attention from the scientific and medical world due to their ability to act as bacterial predators, as bacterial transmission vehicles or a Trojan horse, and notably as biological reservoirs. The ability of amoebae to host bacteria may enhance bacterial infectivity for mammalian cells, thus increasing their transmission to susceptible hosts, as well as enhancing the pathogenicity of the host amoebae; however, the underlying mechanisms remain incompletely understood (Khan 2009; La Scola et al. 2001, 2003; Molmeret et al. 2005; Greub and Raoult 2004). Also, *Acanthamoeba* shares remarkable similarities with macrophages in their phagocytic properties, and it is considered as an environmental phagocyte (Khan 2009). Here, we

determined *Corynebacterium* spp. and *P. aeruginosa* interactions with non-phagocytic HBMEC and phagocytic *A. castellanii*.

It was interesting to note that both *Corynebacterium* spp. and *P. aeruginosa* associated and invaded/taken up by *A. castellanii*, but only *P. aeruginosa* showed higher association and/or invasion of HBMEC. These findings suggest that *Corynebacterium* spp. are weakly pathogenic and are likely taken up by amoeba but unable to invade HBMEC. This is consistent with our findings of cyst survival assays which revealed that *Corynebacterium* spp. exhibited reduced recovery from mature *A. castellanii* cysts compared with *P. aeruginosa*. Amoebae take up bacteria; however, it is not clear whether they are being used as food source. The fact that at least some bacteria are being recovered from cysts suggests that they are able to avoid complete killing by amoeba. Perhaps, this explains the difference between pathogenic and/or weak-pathogenic bacteria who avoid killing, while non-pathogenic bacteria are eaten up by amoeba (Siddiqui et al. 2013; Yousuf et al. 2013). This was confirmed by plating amoeba on non-nutrient agar plates without any food and left for 10 days. During this time, any remaining food bacteria will be eaten up and only the pathogenic ones would be able to remain viable. Overall, these findings suggest that *A. castellanii* cysts act as a reservoir for *P. aeruginosa* and may aid in their transmission to the susceptible hosts, while *Corynebacterium* spp. are weak-pathogenic. These findings corroborate with Ott et al. (2012) who showed that *A. polyphaga* use *Corynebacterium* spp. as food source. Notably, our findings showed that *P. aeruginosa* exhibited higher levels of recovery from mature cysts as compared to *A. castellanii* trophozoite post-invasion counts suggesting that *P. aeruginosa* are able to resist amoebal grazing and multiply within *A. castellanii* cysts. Based on these findings, it is tempting to speculate that *Acanthamoeba* and *P. aeruginosa* are involved in convoluted interactions and suggest their long co-evolutionary history combined with a series of adjustments (including lateral gene transfers) ensuring bacterial survival and that grazing resistance that may have influenced evolutionary gain of *P. aeruginosa* pathogenicity and this will be the subject of future studies. This is strengthened with the fact that *Acanthamoeba* and *P. aeruginosa* are often isolated as co-inhabitants from eyewash stations, drinking water system of hospitals, contact lens cases, biofilms, etc. (Paszko-Kolva et al. 1991; Michel et al. 1995; Greub and Raoult 2004; La Scola et al. 2003). The evolution of one species to house inside another species is a remarkable adaptation and consistent with the fundamental principle of natural selection to favour cooperation. Based on this property, *Acanthamoeba* has often been described as the training ground to learn to evade immune attack (Molmeret et al. 2005). In particular, the ability of bacteria to survive the encystment process may be a useful indicator for bacterial pathogenicity and may aid in the differentiation of pathogenic bacterial species. For example, our previous studies showed that neuropathogenic *E. coli* K1 showed higher recovery from mature cysts but not non-pathogenic K-12 (Jung et al. 2007). Likewise, *P. aeruginosa* (clinical isolate) exhibited higher recovery from mature cysts compared with *Corynebacterium* spp. (environmental isolate). However, this proposal can only be tested for bacterial genus that interact with *Acanthamoeba*.

One of the interesting findings observed in this study was the inability of *P. aeruginosa* to inhibit the growth of *A. castellanii*, even under favourable conditions. Similarly, *P. aeruginosa* conditioned medium had no negative effect on *A. castellanii* growth. This is in contrast to previous studies which showed that *P. aeruginosa* affected *A. castellanii* viability and inhibited growth in a type III secretion system-dependent manner and due to *P. aeruginosa* toxin L-2-amino-4-methoxy-trans-3-butenoic (Wang and Ahearn 1997; Abd et al. 2008; Lee et al. 2012). There are several explanations for these contrasting results, different strains of *A. castellanii* and *P. aeruginosa* used; the strain of *A. castellanii* used in the present study is a clinical isolate belonging to T4 genotype, isolated from a keratitis patient compared with the strain used in the study by Abd et al. (2008) that was derived from a yeast culture, while Lee et al. (2012) used a soil isolate of *A. castellanii*. Alternatively, assay conditions (24 h co-incubation in our assays versus 7 days co-incubation in previous studies) may explain these differences.

Overall, these results showed that bacteria that were highly pathogenic to HBMEC resisted amoebal grazing and survived encystment process with higher yield from mature cysts, compared with bacteria that were weakly pathogenic to HBMEC. Pathogenic bacteria tested have the potential to exploit amoebae cysts as biological vectors; however, the molecular mechanisms of bacterial localization within amoebae, evasion of phago-lysosome processes and inability of amoeba to expel bacteria during encystment remain unclear. Of note, recent studies showed the presence of a diffusible factor produced by amoebae, mediating survival and replication of *B. cepacia and V. parahaemolyticus* (Marolda et al. 1999; Laskowski-Arce and Orth 2008), which may explain our findings. Overall, *Acanthamoeba* acts as an environmental sanctuary for bacterial pathogens, and perhaps facilitating genetic exchanges affecting their virulence, and contributes to microbial survival in harsh environmental conditions along with aiding their transmission to susceptible hosts that is of immense concern for human and animal health. Interaction studies will help to identify the role of *Acanthamoeba* in the evolution of superbugs and in turn expedite discovery of novel therapeutic and/or preventative measures.

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Conflict of interest None to declare.

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