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Prevalence of *Acanthamoeba* spp. in Tasmanian intensive care clinical specimens

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SUMMARY

Background: Acanthamoebae are ubiquitous free-living environmental amoebae that may occasionally cause keratitis, granulomatous encephalitis, cutaneous lesions and systemic disease in humans. *Acanthamoeba* spp. have been implicated as a vehicle by which a number of common bacterial causes of healthcare-associated pneumonia may enter the lungs. Limited evidence has been found implicating *Acanthamoeba* spp. as a primary cause of pneumonia and urinary catheter colonization in intensive care patients.

Aim: To explore the possibility of colonization of the respiratory and urinary tracts of intensive care patients with free-living amoebae.

Methods: Thirty-nine catheter urines, 50 endotracheal trap sputa and one general ward sputum sample from 45 patients and nine intensive care unit (ICU) environmental water samples were collected during a four-and-half-month period in the Royal Hobart Hospital from August 2011.

Findings: Acanthamoebae were isolated by culture and detected by polymerase chain reaction in two sputum samples from a single patient, taken one week apart. A single *Acanthamoeba* species isolate was detected by culture only from the ICU environment.

Conclusion: Colonization of ICU patients' respiratory tracts with *Acanthamoeba* spp. does occur. This may have significance for the role of acanthamoebae as a source of bacterial pathogens in intensive therapy patients' respiratory tracts.

Introduction

Reports of human infection with *Acanthamoeba* spp. have traditionally been limited to cases of systemic disease in the immunocompromised, granulomatous encephalitis, keratitis and cutaneous lesions. In recent years, studies have demonstrated an intrinsic relationship between bacteria and environmental free-living amoebae. Many pathogens important in the intensive therapy setting are capable of survival and even replication within amoebae, particularly *Acanthamoeba*. *Acanthamoeba*-associated organisms include *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Escherichia coli*, *Serratia marcescens*,

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Klebsiella spp. and *Streptococcus pneumoniae*.[1] Acanthamoebae have also been proposed as a reservoir of epidemic strains of meticillin-resistant *Staphylococcus aureus* in the environment.[2] Within environmental amoebae, bacteria are protected from challenges such as heat, cold and disinfection.[2,3] It has been suggested that internalization in acanthamoebae selects for more virulent and more antimicrobial-resistant bacterial strains.[4] It has further been proposed that amoebae are involved in the introduction of disease-causing bacteria to the host, so-called 'Trojan horse theory'.[5] Supporting this theory, murine models have shown that respiratory inoculation with legionella and amoebae leads to far more severe disease than inoculation with the same number of legionella bacteria alone.[6]

Amoebae are prevalent in the hospital environment, having been isolated in water samples from 68.9% of faucets in hospitals, compared with 20–30% in domestic environments.[1] In a French study, 10.5% of 210 cases of healthcare-associated pneumonia were caused with definite amoeba-associated organisms.[7] However, many of the 'negatives' – infecting organisms excluded in this study as not being amoeba-associated – have since been shown to be capable of survival and, in some cases, replication within amoebae.[1] Importantly, in five cases (four healthcare-associated and one community-acquired pneumonia), *Acanthamoeba polyphaga* mimivirus was the only infectious agent identified.[7] Culture for the free-living amoebae themselves from sputa was not undertaken in this study. One year following the above-mentioned pneumonia study, a group from Brazil reported the presence of *Acanthamoeba* spp. in 26% of catheter urine specimens from the intensive care unit of Hospital das Clínicas, University of São Paulo.[8] Culture was performed by adding centrifuged urine to brain–heart infusion broth and incubating for 48 h at 25 °C. Higher urinary leucocyte and erythrocyte counts were noted in *Acanthamoeba* spp.-positive urine samples, suggesting a pathogenic process.[8] This novel finding represented a previously unrecognized niche for colonization of the human body by acanthamoebae. It was postulated that the acanthamoebae recovered formed part of the flora of biofilms coating the patient's urinary catheters.

Predation by free-living amoebae is intrinsically involved in the maintenance of bacterial biofilms in the environment.[9] Therefore, free-living amoebae may have a preference for colonization of substrates which encourage biofilm formation. In the hospital setting, bacterial biofilms are commonly seen forming along plastic tubes such as urinary catheters and endotracheal tubes.[10] If ignored, this biofilm colonization often leads to subsequent bacterial cystitis or pneumonia.[10] This study sought to determine the prevalence of colonization by *Acanthamoeba* spp. of urinary catheters and endotracheal tubes in the intensive care unit (ICU) of the Royal Hobart Hospital (RHH).

Methods

Specimens

Endotracheal trap sputa and catheter urine specimens collected from RHH patients and sent for culture at the RHH microbiology department between August 2011 and January 2012 were included in this study.

Processing of specimens

Sputa were homogenized in 1:1 dilution with sputolysin (Calbiochem, San Diego, Calif.) in Page's saline and spiked with *Escherichia coli* ATCC 25922 prior to culture and polymerase chain reaction (PCR). Urine specimens were made up to 50 mL volume in sterile Page's saline [142 mg Na_2HPO_4 (Merck, Darmstadt, Germany), 136 mg KH_2PO_4 (Sigma–Aldrich, Chemie GmbH, Buchs SG, Switzerland), 120 mg NaCl (Schurlau, Barcelona, Spain), 4 mg $MgSO_4$ (Chem-supply) and 4 mg $CaCl_2$ (Chem-Supply, Port Adelaide, SA, Australia) in 1 L distilled, deionized water, pH adjusted to 6.8 at 25 °C], spiked with *Escherichia coli* ATCC 25922 and then centrifuged at 2500 rpm for 5 min. The urine deposit was used for culture and PCR. Water samples (200 mL) were spiked with *Escherichia coli* ATCC 25922 and centrifuged at 2500 rpm for 5 min. The resultant deposit was then separated for culture and PCR. A positive control consisting of a clinical keratitis isolate of *Acanthamoeba* sp. was inoculated into 50 mL of Page's saline, and then treated as a urine specimen and incorporated into each set of cultures performed. Negative control consisted of 50 mL of Page's saline alone, processed as per a urine specimen.

Culture for Acanthamoeba species

One drop (~20 μL) of urine/water/control deposits and sputa for amoeba culture was expressed on to the centre of non-nutrient agar plates [15 g of bacteriological agar (Oxoid Thermo-Fisher, Scientific, Thebarton, SA, Australia) in 1 L of Page's saline] and overlaid with a microscope coverslip. Agar plates were incubated at 30 °C in air for 72 h. Following incubation, the coverslip was transferred on to a clean microscope slide with a drop of sterile water and examined under ×100 and ×400 magnification for cysts and trophozoites of amoebae.

Culture for Legionella species

Unprocessed sputum specimen was inoculated on to CYE, CYE MWY and CYE BMPA agar for *Legionella* culture (Biomeriéux, Marcy-l'Étoile, France) and incubated in air for seven days, being analysed for colonies morphologically resembling *Legionella* species on days 5 and 7. *Legionella* culture was not performed on environmental water samples.

PCR detection of Acanthamoeba species

Urine/water deposits and sputa for PCR were stored at −20 °C prior to processing. PCR was performed in runs of up to 20 samples at a time. Specimens were boiled at 100 °C for 10 min to liberate DNA from cysts and trophozoites, then 2 μL used for PCR. Real-time PCR for *Acanthamoeba* spp. was performed using a StepOne PCR cycler (Applied Biosystems, Foster City, Calif.) according to the method described by Qvarnstrom *et al*. with two modifications: primers and probes for *Naegleria fowleri* and *Balamuthia mandrillaris* were not included, and the modified cycling conditions described by Chang *et al*. were used.[11,12] The limit of detection (LOD) was determined by using a solution of cysts counted in a cell counting chamber, then diluted 1:10 with sterile water until a PCR product was not detected. LOD assays were performed in triplicate. All specimens were tested for PCR inhibition by repeating the PCR of the sample after it was spiked with positive control DNA.