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Acanthamoebae as a protective reservoir for *Pseudomonas aeruginosa* in a clinical environment

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SUMMARY

Background: *Pseudomonas aeruginosa* is a growing concern in healthcare-associated infections and poses significant risk to those with serious underlying health conditions. The antimicrobial resistance traits of the pathogen and ability to form biofilms make effective mitigation and disinfection strategies difficult. Added to this challenge is the role that free-living amoebae such as *Acanthamoeba* play in the detection, disinfection and transmission of *P. aeruginosa*. *P. aeruginosa* can survive intracellularly within amoebae, which has the potential to limit detectability and permit transmission into high-risk areas.

Methods/findings: We screened for the presence of *Acanthamoeba* spp. and *P. aeruginosa* within a functioning general hospital in Scotland using a culture and molecular approach, noting their presence at several sites over a four-month period, particularly within floor drains connecting patient rooms. In addition, microbiome analysis revealed that amoebae harbour a unique microbial community comprised primarily of *Pseudomonas* spp. that were not readily detected using microbiome sequencing techniques on environmental swabs. Having demonstrated that both organisms were consistently present in hospital settings, we investigated the relationship between acanthamoeba and *P. aeruginosa* in the laboratory, showing that (i) acanthamoeba growth rate is increased in the presence of pseudomonas biofilms and viable pseudomonas persist within the amoebae and (ii) hydrogen peroxide-based disinfectants are significantly less effective against an isolate of *P. aeruginosa* in the presence of acanthamoeba than when the bacteria are incubated alone.

Conclusions: These findings suggest that amoebae, and other protists, can influence the detection and persistence of *P. aeruginosa* in high-risk areas and should be considered when implementing mitigation strategies.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of humans. At particular risk are people who have serious underlying health conditions such as cystic fibrosis and patients who are in augmented care units [1,2]. *P. aeruginosa* is documented as having a prevalence of 7.1—7.3% in healthcare-associated infections [3—5], with this number increasing to 16.2% in intensive care unit (ICU) patients [5,6]. Treatment is limited due the broad intrinsic resistance of the bacteria and its ability to acquire or develop resistance traits through gene transfer events or chromosomal mutations [7]. Outbreaks have been linked to water sources or situations where high moisture levels are found within the healthcare setting [8,9]. The outcome of an independent review of *P. aeruginosa* outbreaks in neonatal units in Northern Ireland recognized and reported on the role of water distribution systems in the spread of infection and made specific recommendations about the potential risk from contaminated taps and use of sinks [8]. In this incident four neonates died. Thus, a comprehensive approach to monitoring these systems is essential.

We have previously worked with *P. aeruginosa* in the context of device-related disinfection and have demonstrated that environmental factors, including biofilm formation, have the potential to drive disinfection efficacy [10]. Factors that are less considered, however, are the consequences of interactions between *P. aeruginosa* and other organisms within their natural ecosystems such as predatory protists (e.g., free-living amoebae). Acanthamoeba are a genus of free-living amoebae, ubiquitous in the environment and highly efficient predators of bacteria, engulfing and feeding via phagocytosis [11,12]. However, some bacteria such as *P. aeruginosa* have evolved strategies that prevent phagocytic breakdown within the amoebae and instead permit intracellular survival [13—15]. The risk of this vector-like association to human health is unknown but there is evidence to suggest that bacteria existing within the amoebae are less readily detected, capable of surviving at higher temperatures and antimicrobial concentrations, and potentially capable of developing or acquiring drug-resistant traits at an increased rate [15—17]. For example, *Legionella pneumophila* internalized by acanthamoeba are capable of surviving in chlorine concentrations four-fold higher relative to extracellular cells and at temperatures as high as 90 °C [16,17]. Similar findings for chlorine disinfection against *P. aeruginosa* have been reported by Sarink and colleagues [18]. Additionally, viable but non-cultivable (VBNC) *P. aeruginosa* cells can be 'resuscitated' in the presence of *Acanthamoeba polyphaga* in as little as 2 h [13], posing increased risk to human health. These observations suggest that interactions between these two species are complex and further work by Corsaro and colleagues demonstrated that pathogenic bacteria, including *P. aeruginosa*, could persist within free-living amoebae thus avoiding disinfection within water distribution systems [19]. Persistence within *Acanthamoeba* spp. may well drive adaptation of *P. aeruginosa* towards human infection. Leong and colleagues reported finding single-nucleotide repeats in the genome of *P. aeruginosa* after prolonged persistence in *Acanthamoeba castellanii* leading to reduced virulence, enhanced fitness, and enhanced survival in macrophage and neutrophils, similar to strains recovered from cystic fibrosis patients [20].

Healthcare settings and hospitals, in particular, care for individuals with greater susceptibility to infection and reduced immune function. Such individuals are prone to infection by opportunistic pathogens such as *P. aeruginosa*. Free-living amoebae have been found in drinking water systems [21] and isolated within healthcare settings [22—24], opening the possibility that interaction between *Acanthamoeba* spp. and *P. aeruginosa* could play a role in the distribution, persistence and survival of *P. aeruginosa* in healthcare settings. Herein, we further elucidate the risks of this relationship within high-risk areas, both in the detection of the pathogen and in its effective disinfection.

Methods

Sample collection and storage

Sample collection for this study was undertaken over a four-month period in a functioning general hospital within Scotland with permissions. Hospital water samples were collected in sterilized bottles, approximately 1 L per sample. Swabs were also taken from surfaces, swabbing each surface for a total of 10 s to ensure the entirety of the swab made contact, before placing into sterile phosphate-buffered saline (PBS). A total of four sampling events were conducted. Sampling events 1—3 gathered samples from five locations: water tank, sink, showerhead, tap head and floor drain. Sampling event 4 gathered samples from the floor drain only. All sampling apart from the water tank was carried out in clinical areas.

Extraction of genomic DNA and molecular screening using polymerase chain reaction

DNA was extracted using the QIAmp Fast DNA Stool kit using a modified protocol [25]. Prior to extraction, 1 L of water was filtered through a 0.22-μm filter and DNA extracted directly from the filter; or for swabs, DNA was extracted directly from the swab and PBS. Upon elution, DNA was quantified using the Qubit 4 (Invitrogen) and stored at −20 °C prior to downstream use. Successful extractions were screened by polymerase chain reaction (PCR) using the Expand Hi-Fidelity PCR kit (Merck), as per the manufacturer's instructions, to determine the presence of *P. aeruginosa* and *Acanthamoeba* spp. using species- and genus-specific primers, respectively: *P. aeruginosa* F 5′-GGGGGATCTTCGGACCTCA-3′ and R 5′-TCCTTAGAGTGCCCACCCG-3′ [26], *Acanthamoeba* spp. JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′ [27].

Isolation of Pseudomonas species and free-living amoebae from samples

Hospital surfaces and water samples were assessed for the presence of *Pseudomonas* spp. by directly streaking swabs from sinks, shower heads, floor drains and tap heads on 0.3% w/v cetrimide agar (CA) or passing water through filters and culturing on CA to recover *Pseudomonas* spp. after which the plates were incubated at 37 °C for 24 h. One litre of each water sample taken for the sink and water tank were filtered through a 0.45-μm PVDF sterile filter after vigorous agitation for homogeneity and this filter was then placed directly on to CA; this was performed in triplicate for each water sample.

Culturable free-living amoeba (FLA) species were isolated from samples by filtering 500 mL of water through a 0.45-μm filter and placing the filter on non-nutrient amoeba saline agar (NN-agar) [28] or by direct streaking of the swab. Amoebae were identified microscopically before being placed on a fresh NN-agar plate and allowed to migrate across a heat-killed *Escherichia coli* gradient. Upon isolation, samples were maintained as trophozoites using a modified media (minimal amoebic detection (MAD) media; potassium dihydrogen orthophosphate 360 mg/L, methionine 300 mg/L, salt solution 1 ml/L (stock salt solution: $CaCl_2.2H_2O$, 150 mg; $FeCl_3$, 20 mg; $MgSO_4.7H_2O$, 2.46 g; distilled H_2O, 100 mL), thiamine 1200 mg/L, arginine-HCl 825 mg/L, biotin 16.66 μg/L, B12 8.33 μg/L, serine 1050 mg/L, lysine 1250 mg/L, aspartic acid 750 mg/L and distilled H_2O) to limit bacterial or fungal growth and incubated at 25 °C for one to two weeks. Morphological identification of the isolated acanthamoeba-like organisms was based on both trophozoite and cyst morphology before being confirmed through PCR amplification using genus-specific primers JDP1 and JDP2.

Microbiome sequencing

Microbiome analysis was carried out using the 16S rRNA gene universal primers specific to the conserved region V1–V3 to assess the shift in the overall population dynamics. Samples were sequenced using an Illumina platform. Chimeric reads were removed using the VSEARCH package [29]. High-quality reads were partitioned into operational taxonomic units (OTUs) using minimum entropy decomposition [30,31] and DC-MEGABLAST was used for taxonomic assignment. The lowest taxonomic unit for each OTU was then assigned. A sequence identity of 70% across at least 80% of the representative sequence was a minimal requirement for considering reference sequences. Further processing of OTUs and taxonomic assignments was performed, and Alpha (Shannon index) and Beta (Bray–Curtis dissimilarity) diversity metrics were generated using the QIIME software package (version 1.9.1, http://qiime.org/). Abundances of bacterial taxonomic units were normalized using lineage-specific copy numbers of the relevant marker genes to improve estimates [32].

In vitro *culture of* Pseudomonas *spp. and A. castellanii*

Culture of Pseudomonas spp.

P. aeruginosa NCTC 10332 and *Pseudomonas putida* mCherry [33] were cultured in Luria Broth (LB; Oxoid) and 0.3% w/v Cetrimide Agar (Oxoid) and quantified by turbidity of centrifuged, washed and resuspended cells (OD 0.2 value at 600 nm approximating 10^9 cfu/mL).

Biofilm formation

Biofilms of *P. aeruginosa* were prepared in microtitre plates in full LB, 10% LB/90% autoclaved tap water, and filter-sterilized tap water at temperatures of 25 and 37 °C for seven days. Biomass was assessed at days 1, 3, 5 and 7 by Crystal Violet assay, using 70% v/v ethanol dissolution and measuring absorbance at 570 nm with a Tecan plate reader (Tecan Infinite F200 Pro).

Culture of A. castellanii

A. castellanii (ATCC 50370) were grown under sterile conditions in peptone-glucose (PG) media [34] at 25 °C. Trophozoites were maintained in PG media prior to experimental work. Stationary phase trophozoites were converted to cysts by removing PG media, washing once with PBS and adding encystment media [34]. Cells were then incubated for one week at 25 °C to allow complete encystation.

Cytotoxicity work

P. aeruginosa, *P. putida* mCherry and *A. castellanii* trophozoites and cysts were exposed to various concentrations of a commercial preparation of silver-stabilized hydrogen peroxide (SS-H_2O_2), 5% (w/w) H_2O_2 with trace silver. The minimum inhibitory concentration was observed after incubating with SS-H_2O_2 overnight at 37 °C (*Pseudomonas*) or exposing to SS-H_2O_2 for 30 minutes, washing and incubating for 25 °C (*Acanthamoeba*). Both *Pseudomonas* species were seeded at a density of 1×10^5 cfu/mL and incubated with or without SS-H_2O_2 (25,000 ppm–0.1 ppm). Recovery was measured by absorbance at 600 nm (Tecan Infinite F200 Pro). Similarly, *A. castellanii* trophozoites and cysts were seeded at a density of 8×10^4 cells/mL and given 1 h to adhere to the plate surface. Cells were then treated with or without the SS-H_2O_2 solution (25,000 ppm–0.1 ppm) for 30 min. Media were then removed from each well and centrifuged to recover planktonic cells before being resuspended in PG media and placed back into the relevant wells. Trophozoites and cysts were given 24 h and one week to recover, respectively, before viability was assessed using Alamar blue as described previously [35].

A mixed species assay was undertaken using both *A. castellanii* and *P. aeruginosa*. *P. aeruginosa* were seeded at a density of 1×10^5 cfu/mL with or without *A. castellanii* trophozoites (8×10^4 cells/mL). Amoebae were given 1 h to engulf bacteria prior to the addition of the SS-H_2O_2 solution. Samples were then exposed for 30 min before removal. LB was subsequently added to each well and incubated at 25 °C for 18 h before placing at 37 °C for 24 h. Media from each well were then placed into a new well for analysis. Acanthamoeba trophozoites remained adhered to the original well surface under all conditions (verified microscopically), allowing *P. aeruginosa* density to be assessed by absorbance at 600 nm (Tecan Infinite F200 Pro).

Results

Pseudomonas *spp. and* Acanthamoeba *spp. presence in hospital systems*

Swab and water samples were collected from several high-risk areas within a Scottish hospital that are subject to regular disinfection: water storage tank, patient sink, patient showerhead and patient tap head. Additionally, samples from the floor drain within the patient's room were also taken. Four sampling events were carried out within a four-month period. These sites were screened for the presence of *Acanthamoeba* spp. and *P. aeruginosa* using a culture and molecular approach (Table I). Acanthamoeba isolates were morphologically identified by the characteristic

Table I
Culture and molecular screening of multiple sites within an unnamed Scottish hospital for the presence of *Acanthamoeba* spp. and *Pseudomonas* spp.

Sample	Culture positive FLA				Culture positive *Pseudomonas* spp.				Culture-positive *Pseudomonas aeruginosa*				PCR-positive *Acanthamoeba spp.*				PCR-positive *P. aeruginosa*			
	Sampling event																			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Water tank (W)																				
Sink (W)																				
Sink (S)																				
Showerhead (S)																				
Tap head (S)																				
Floor drain (S)																				

PCR, polymerase chain reaction; S, swab; W, water. Red box = negative, green box = positive, grey box = not screened.

PCR, polymerase chain reaction; S, swab; W, water. Red box = negative, green box = positive, grey box = not screened.

cyst structure and *P. aeruginosa* isolates by meeting the following criteria: Gram-negative, tolerant of cetrimide, oxidase positive, catalase positive, and capable of growing at 41 °C. Furthermore, DNA was extracted from swabs, filtered water and cultured isolates and screened using the acanthamoeba genus-specific JDP primers [27] and *P. aeruginosa* specific primers [26].

Culture based results found no evidence of *Pseudomonas* spp. at any site with the exception of the floor drain (Table I). Of all culturable *Pseudomonas* spp., only one sampling event (Table I; sampling event 2) contained organisms that met the necessary criteria for classification as *P. aeruginosa*. FLA were observed in and around the sink and tap areas during sampling event 3 and frequently in the floor drain (Table I). Molecular screening for *Acanthamoeba* spp. and *P. aeruginosa* found evidence of both at the sink area during sample event 1 and in the floor drain during sample events 1, 2 and 4 (Table I). The frequency of these organisms in the floor drain microbiome poses a potential risk given the potential for FLA to act as migratory vectors of known pathogens, such as *P. aeruginosa*, and the interactions of these organisms within this system warrant further consideration.

16S rRNA profile of the overall floor drain microbiome relative to the intracellular microbiome of FLA from the same system

The total genomic DNA from the floor drain was extracted (Figure 1; extracellular) as was the DNA from the isolated *Acanthamoeba* spp. (Figure 1; intracellular) at the same site. Prokaryotic profiling was achieved by next-generation sequencing of the 16S rRNA gene, region V1—V3, and the lowest taxonomic unit (LTU) assigned. A significant variability can be observed between the sites with the total gDNA sequences dominated primarily by anaerobic bacteria such as *Olsenella* spp. (Figure 1a), of which some members are capable of causing infections in humans, e.g., *Olsenella uli* [36]. Interestingly, the total gDNA from isolated *Acanthamoeba* spp. including that of its microbiome was dominated by *Pseudomonas* spp. and other Gram-negative aerobes (Figure 1a) with *Pseudomonas* spp. accounting for 56% of all reads.

The extracellular samples revealed a higher diversity than the intracellular samples (Figure 1a), with Shannon index scores of 2.94 and 1.88, respectively. Of the assigned OTUs, there was an almost even split of known Gram-negative and Gram-positive organisms in the extracellular sample (Figure 1b: 60%—40% for negative and positive, respectively); however, only Gram-negative organisms were detected from the intracellular samples (Figure 1b). The degree of diversity between samples was further evidenced by the level of unique OTUs observed, with only one shared OTU detected (Figure 1c: Rhizobiaceae) and a Bray—Curtis dissimilarity score of 1. We demonstrate here that protists such as FLA can harbour a unique microbiome which can go undetected using current microbiome sequencing techniques and could act as a vector for potential pathogens such as *P. aeruginosa* in high-risk areas.

Intracellular survival of Pseudomonas *spp. in* A. castellanii

Acanthamoeba species are bacterivores, feeding on bacteria via phagocytosis [11,12]. We found that incubation of *A. castellanii* on *P. aeruginosa* biofilms resulted in a 5.3-fold increase in A. *castellanii* cell density. However, *Pseudomonas* spp. are known phagocyte-resistant organisms and as such can overcome the predatory behaviours of *Acanthamoeba* spp. by surviving intracellularly [13—15]. To demonstrate the fate of biofilm associated *Pseudomonas* spp. when incubated with *A. castellanii* trophozoites, 1×10^4 trophozoites were seeded on to an mCherry labelled *Pseudomonas putida* biofilm and the fate of fluorescent bacteria monitored over 72 h (Figure 2). A visible reduction of the biofilm was observed relative to a

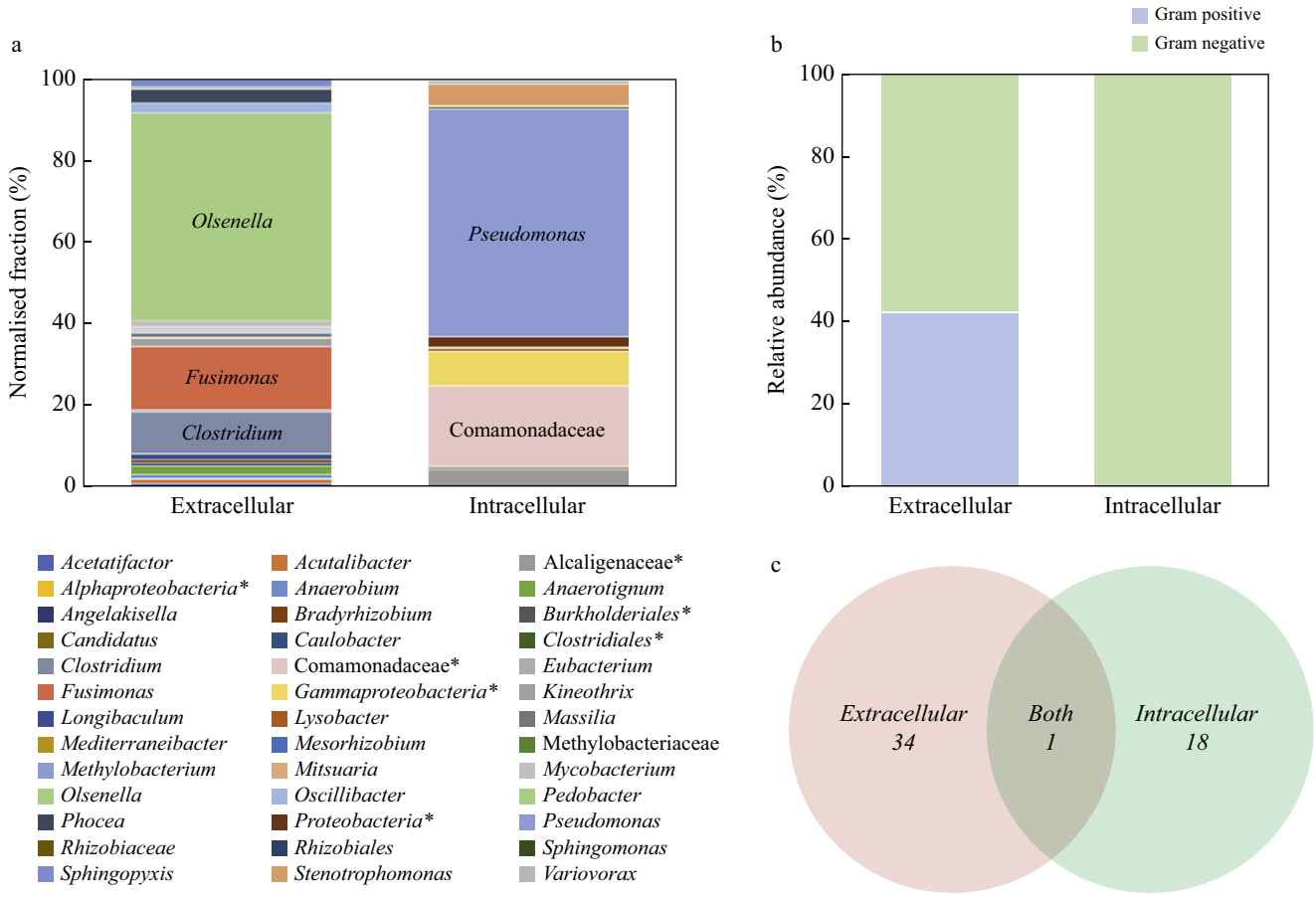

Figure 1. Molecular characterization of extracellular and intracellular prokaryotes. Total genomic DNA (extracellular) was isolated from floor drain swabs and isolated amoebic cultures (intracellular) and microbiome sequencing of the 16S rRNA gene region V1–V3 undertaken to determine prokaryotic diversity. (a) Sequence reads were assigned to the genus level where possible, operational taxonomic units (OTUs) above genus level are denoted by *, and abundance estimated using a normalized fraction of the OTU sequence reads. Taxonomic assignments for highly abundant reads (>10%) are noted within their respective data bar. Figure key shows combined taxonomic assignments for both conditions. (b) Spread of organisms as determined by known Gram classification. (c) Total number of unique and shared OTUs for each sample.

biofilm-only control (Figure 2a compared with 2c). Notably, a shift from a non-specific localization of *P. putida* cells at day 0 to localization within amoebae vacuoles after day 3 suggests that the bacteria were internalized by the amoebae (Figure 2b compared with 2c).

Inhibitory effect of silver-stabilized hydrogen peroxide on P. aeruginosa *in the presence of* A. castellanii

To determine the role of acanthamoeba in protecting *Pseudomonas* spp. from antimicrobial compounds, a co-incubation assay was undertaken using *A. castellanii* trophozoites (8×10^4 cells/mL) and *P. aeruginosa* (1×10^5 cfu/mL). *P. aeruginosa* were incubated for 1 h with or without *A. castellanii* trophozoites (Figure 3; green and blue bars, respectively) before being exposed to three concentrations of SS-H_2O_2 (400 ppm, 800 ppm and 1600 ppm). Control wells using *P. aeruginosa* and *A. castellanii* individually were clear at all concentrations of SS-H_2O_2 (Figure 3; green and red bars, respectively). Interestingly, in wells containing both *A. castellanii* and *P. aeruginosa*, a significant increase in turbidity was observed below the *A. castellanii* trophozoite minimum inhibitory concentration (MIC) (400 ppm and 800 ppm) relative to *P. aeruginosa* alone (Figure 3; blue bars relative to green bars, $P<0.01$). These results demonstrate that *A. castellanii* can protect *P. aeruginosa* from disinfection. Growth of *P. aeruginosa* was possible at concentrations 64-fold higher than required to inhibit growth in planktonic monocultures when the bacteria were incubated for 1 h with *A. castellanii* trophozoites (Supplementary Figure S2). It is also worth noting here that the survival of the bacteria appears to be reliant on the survival of *A. castellanii*, with no bacterial regrowth observed at MICs for acanthamoeba (Figure 3; blue bar, 1600 ppm; amoebic MIC shown in Supplementary Figure S2).

Discussion

While research into the impact of microbial interkingdom relationships is increasing, the consequences of these interactions with regards to human health remains relatively

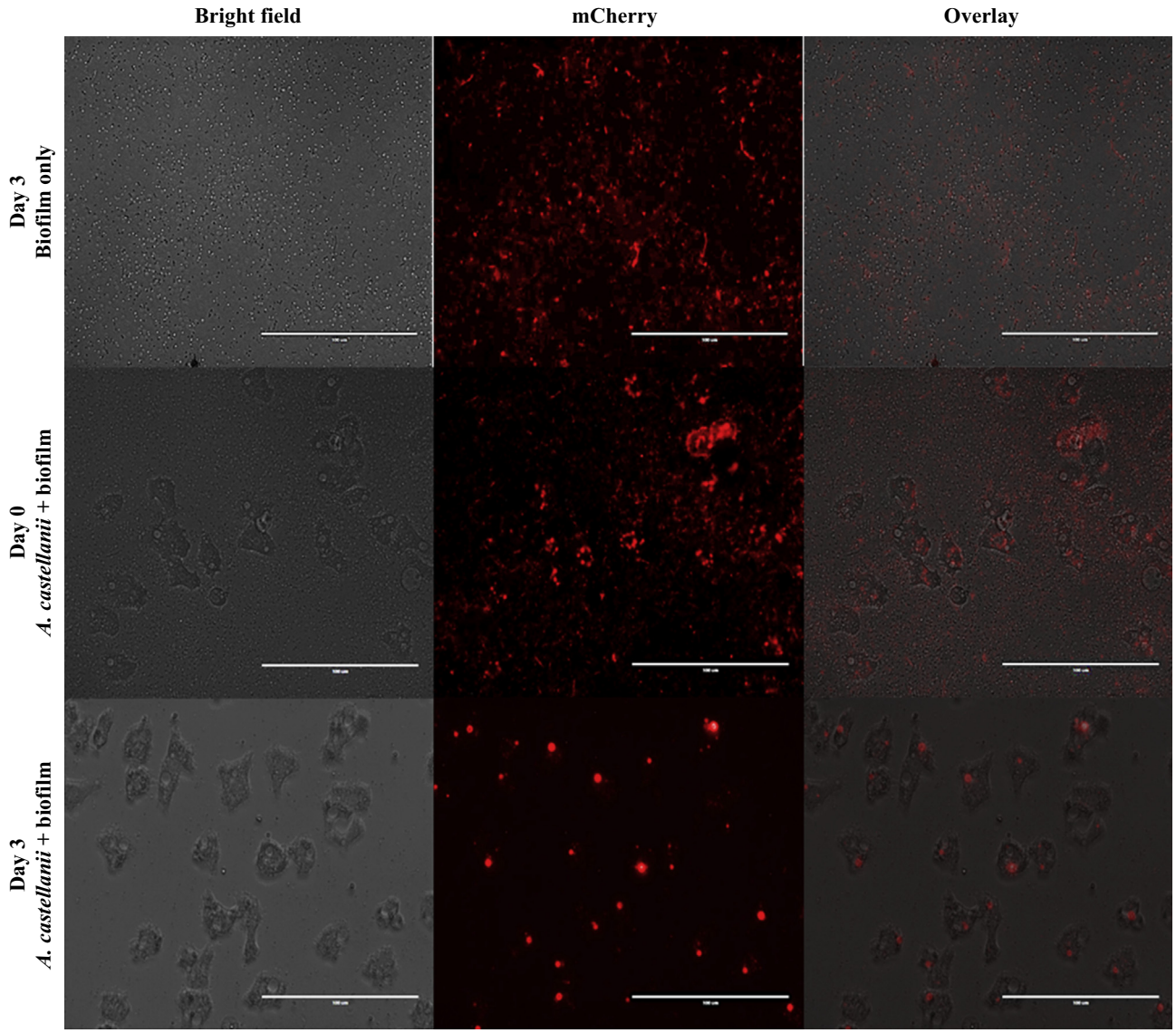

Figure 2. Fate of biofilm-associated *Pseudomonas putida* mCherry in the presence of *Acanthamoeba castellanii* trophozoites: *P. putida* mCherry biofilms were incubated for three days with or without *A. castellanii* trophozoites (1×10^4 cells/mL) and their localization within cultures monitored by fluorescent microscopy. After three days of incubation with *A. castellanii*, fluorescence appears exclusively within the vacuoles of the amoebae and not outside as is observed on day 0.

unexplored. *P. aeruginosa* is a significant clinical concern and the implementation of effective detection and mitigation strategies in high-risk areas is paramount. We have demonstrated that, as part of these efforts, consideration must be given to the wider microbiome and the interactions of other organisms with potential pathogens such as *P. aeruginosa*. Our results found that both *P. aeruginosa* and *Acanthamoeba* spp. can be readily detected in high-risk areas such as hospitals. However, detection using standard approaches can be inconsistent and can fail to detect intracellular *Pseudomonas* spp. In addition, we have shown that the relationship between *Acanthamoeba* and *Pseudomonas* spp. can influence antimicrobial susceptibility and growth rate.

As part of this research, we screened for the presence of *Pseudomonas spp.*, *P. aeruginosa* and *Acanthamoeba* spp. using both culture-based and molecular approaches in several sites within an unnamed Scottish hospital over four sampling events. While we were able to identify *Acanthamoeba* spp. at four of the sites (sink water, sink swab, tap head and floor drain), *P. aeruginosa* was only identified using PCR in the sink water and floor drain. Disinfection and cleaning processes may explain the variability between sample events and sites. In areas like tap heads, which are more accessible, cleaning and disinfection may be easier, when compared to floor drains for example. DNA extracted from swabs taken at the hospital floor-drain showed anaerobic bacteria to dominate the environment, perhaps indicative of the time in which the biofilm had been undisturbed. Contrastingly, isolation of *Acanthamoeba* from the site into a new sterile environment resulted in a major shift to a microbiome comprised almost entirely of unique genera. Genomic DNA extracted from the isolated acanthamoeba was dominated by *Pseudomonas* spp., however other potentially pathogenic genera were also detected, such as *Stenotrophomonas*. It is worth noting that many amoebae can form a

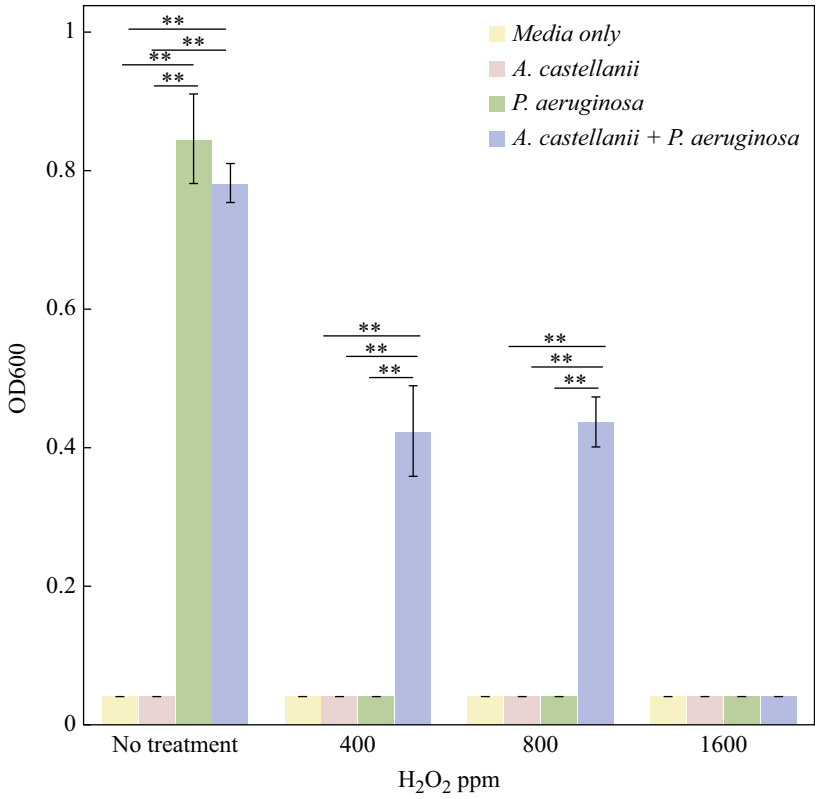

Figure 3. Protective role of *Acanthamoeba castellanii* on *Pseudomonas aeruginosa* in response to silver-stabilized hydrogen peroxide. Optical density of LB media (yellow bar), *A. castellanii* trophozoites monocultures (8 × 10^4 cells/mL, red bar), *P. aeruginosa* monocultures (1 × 10^5 cfu/mL, green bar) and *A. castellanii* and *P. aeruginosa* co-cultures (blue bars) was measured upon 30 min treatment with or without three concentrations of silver-stabilized hydrogen peroxide (400, 800, and 1600 ppm) following two incubation periods (18 h at 25 °C followed by 24 h at 37 °C). Significant growth of *P. aeruginosa* was observed when exposed to 400 ppm and 800 ppm relative to all other conditions ($P<0.01$). One-way analysis of variance (ANOVA) $P<0.01$ as denoted by **, data are shown as mean ± standard deviation; $N=3$ independent experiments performed in triplicate.

highly resilient cyst stage when faced with unfavourable conditions caused by exposure to antimicrobials or disinfectants [34,37,38]. These cysts are significantly more resistant to disinfection than the active trophozoite stage and can remain dormant for years in wait for optimal conditions [39]. Thus, it is possible that *Acanthamoebae* cysts in these environments could be shielding intracellular bacteria from environmental pressures and limiting detection capabilities. Due to the ability of amoebae to harbour potential pathogens [15—17,23,40,41], it is becoming increasingly evident that effective mitigation and detection strategies should consider these, and similar protists, as potential vectors of disease. While culture-based methods remain an important metric for monitoring key pathogens, the data demonstrated within this work serve as a reminder that utilizing a combined molecular and culture-based approach is essential to maximize monitoring coverage and minimize patient risk. Furthermore, it is worth considering that culture-based and molecular screening could be widened to encompass associated organisms such as acanthamoeba that have the potential to conceal pathogenic organisms and pose an underlying risk post-disinfection.

The ability of planktonic *Pseudomonas* spp. to survive inside *Acanthamoeba* spp. has been well established [13]. However, the interaction of amoebae with *Pseudomonas* spp. biofilms is less understood. It is largely considered that the emergence of the amoeboid cell type was due to the selective advantages it provides in relation to biofilm grazing [42], and studies have shown that amoebae are capable of significantly influencing biofilm composition [43]; however it is unclear as to what impact the resistance of *Pseudomonas* spp. to amoebic predation plays on the grazing activity of amoebae on *Pseudomonas* spp. biofilms. Interestingly, our results found that *Acanthamoebae* and *Pseudomonas* spp. existed in an almost mutualistic relationship, with *Acanthamoebae* capable of using the biofilm as a food source, increasing *Acanthamoeba* spp. cell density by 5.3-fold relative to the no-biofilm control, before going on to act as a vector for the internalized bacteria. However, the exact nature of this relationship requires further investigation.

Finally, *Acanthamoeba* spp. are significantly more resistant to many antimicrobials than planktonic *Pseudomonas* spp. We have shown that the resistance of *A. castellanii* to SS-H_2O_2, a hydrogen peroxide-based disinfectant, could be extended to the intracellular bacteria, with *P. aeruginosa* capable of surviving in the presence of concentrations 64-fold higher than the minimum inhibitory concentration of *P. aeruginosa* alone. This protective role is concerning and raises questions on the efficacy of current disinfection strategies against mixed microbial communities. An additional noteworthy observation that was not investigated within this work is what role amoebae play in

the transformation of bacteria from VBNC to culturable states upon treatment with SS-H_2O_2, or indeed other oxidizing agents known to trigger this conversion (e.g., hypochlorous acid). It has been shown that these compounds can cause a shift to non-culturable states [44—49], while amoebae have been shown to facilitate transformation back to the culturable state [13,16]. Thus, it is conceivable that, in addition to providing a protective role, FLA such as *Acanthamoebae* could act as catalysts for the re-emergence of pathogenic bacteria post-disinfection in high-risk environments such as hospitals.

In conclusion, given the capabilities of *P. aeruginosa* to readily form biofilms that permit survival in unfavourable conditions [50] alongside the high propensity for acquiring antimicrobial resistance traits [7], the ability to utilize amoebae as an alternative resistance mechanism is concerning. We have demonstrated here that amoebae are present in high-risk areas and have a close association with *Pseudomonas* spp. Our results have further demonstrated the protective role of amoebae against effective disinfection of *P. aeruginosa* and suggest that the implementation of effective mitigation strategies should account for the increased tolerance of these vectors to ensure risk is minimized. Similarly, the routine screening for these organisms should consider the reduced detectability of intracellular bacteria and the role of amoebae as potential vectors of disease. Our results have demonstrated the risk that protist—bacterial interactions could pose in healthcare settings, and further emphasize that the adoption of a 'One Health' approach to effectively monitor and mitigate the risk of pathogenic organisms in high-risk areas is a necessity.

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Conflict of interest statement
The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2024.08.010.

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