



Short Report

Acanthamoeba castellanii interferes with adequate chlorine disinfection of multidrug-resistant *Pseudomonas aeruginosa*

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SUMMARY

Verona-Integron-encoded-Metallo- β -lactamase-positive *Pseudomonas aeruginosa* (VIM-PA) is a cause of hard-to-treat nosocomial infections, and can colonize hospital water networks alongside *Acanthamoeba*. We developed an in-vitro disinfection model to examine whether *Acanthamoeba castellanii* can harbour VIM-PA intracellularly, allowing VIM-PA to evade being killed by currently used hospital disinfectants. We observed that *A. castellanii* presence resulted in significantly increased survival of VIM-PA after exposure to chlorine for 30 s or for 2 min. This undesirable effect was not observed after disinfection by 70% alcohol or 24% acetic acid. Confocal microscopy confirmed the presence of VIM-PA within *A. castellanii* pseudocysts. Our data indicate that *A. castellanii* contributes to persistent VIM-PA colonization of water systems after chlorine treatment.

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Introduction

Multidrug-resistant strains of *Pseudomonas aeruginosa* (PA) are an important cause of nosocomial infections and are involved in hospital outbreaks worldwide [1]. PA infections are associated with increased morbidity and mortality in hospitalized patients [1]. Furthermore, if these bacteria harbour a Verona Integron-encoded Metallo- β -lactamase (VIM) gene, treatment options are even more limited, as VIM-producing PA can hydrolyse all classes of β -lactams except monobactam

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antibiotics, resulting in resistance against the carbapenems, an important class of antibiotics for treatment of PA infections.

PA can be found in moist environments, such as water networks, which are well-known environmental sources of PA outbreaks in hospitals [2]. Transmission within hospitals occurs through unidentified and presumably persistent sources that are most likely in the water distribution systems or wastewater drains [3,4]. Despite extensive infection prevention measures, PA is in many settings still able to spread [3,5]. Like PA, *Acanthamoeba castellanii* is also present in hospital water networks. These protozoa are known to co-occur with PA at the same locations, and PA were shown to be present intracellularly in *Acanthamoeba* spp. isolated from a hospital water system [6,7]. This phenomenon was confirmed *in vitro*, as phagocytosed PA were shown to remain viable during encystation of *Acanthamoeba* trophozoites [8]. *Acanthamoeba* cysts are known to be resistant to several disinfection treatments used in healthcare settings [9]. Therefore, we hypothesized that PA could survive in hospital environments and resist disinfection by being concealed within *A. castellanii*.

Methods

Strains and materials

Acanthamoeba castellanii ATCC strain 30010 ('Neff') was grown in cell culture flasks at 25°C in PYG medium, which contained proteose peptone, yeast extract, glucose, salt additives (ATCC medium 712), 40 µg/mL gentamicin, 100 units/mL penicillin, and 100 µg/mL streptomycin. All experiments were started with trophozoites growing in logarithmic phase. The PA strain used in all experiments (VIM-PA-R15111) harboured a VIM gene, was isolated from a sink in our hospital, and had caused an outbreak that was recently described [3]. VIM-PA was grown to stationary phase in tryptic soy broth, after which glycerol stocks were prepared by addition of glycerol to a final concentration of 10% (v/v). Stocks were stored at –20°C until use and bacterial density was determined by plating on blood agar plates (BD Diagnostics, Breda, The Netherlands).

The 1000 ppm chlorine disinfection solution was prepared directly before each experiment by addition of 1.5 L lukewarm tap water to an Ecolab (St Paul, MN, USA) Medicarone tablet, resulting in sodium dichloroisocyanurate as a source of free active chlorine. Ethanol (70%, v/v) was purchased from Fresenius Kabi (Bad Homburg, Germany) and 24% (v/v) acetic acid was prepared by dilution of 96% acetic acid (Merck, Kenilworth, NJ, USA) in distilled water.

In-vitro disinfection

A. castellanii trophozoites were collected by placing a cell culture flask on ice for 20 min and repeatedly tapping to detach trophozoites. The contents of the cell culture flask were transferred to a 50 mL tube and centrifuged at 1000 g, after which the pellets were washed once with phosphate-buffered saline (PBS). The VIM-PA glycerol stocks were thawed and diluted in PBS. Co-culture experiments were prepared in 24-well plates by addition of 10⁶ colony-forming units (cfu) VIM-PA to 1 mL PBS with or without 25,000 *A. castellanii* trophozoites, corresponding to a multiplicity of infection of 1:40. After incubation at 25°C for 14 days, the 24-well plate was

placed on ice for 20 min, after which the well contents were collected into separate tubes by vigorous pipetting. The collected material was centrifuged at 1000 g, after which the supernatant was discarded, and the pellet was treated with 1 mL of various disinfectant solutions. Samples were then immediately and intermittently vortexed to ensure that dispersed cells were exposed to the disinfectant. Exposure to chlorine was neutralized after 30 s, after 2 min, or after 5 min by adding sodium thiosulfate (Sigma, St Louis, MO, USA) to obtain a final concentration of 4 mg/mL, which converts chlorine to chloride, thereby ending chlorine treatment. The effect of ethanol was abolished after 30 s or after 2 min by dilution with 9 mL PBS. After centrifugation, the chlorine- and ethanol-neutralized samples were washed three times with 1 mL PBS. Acetic acid was removed after 30 min by washing three times with 1 mL PBS. Exposure times were chosen based on infection prevention guidelines on disinfection, manufacturers' instructions, or literature [10,11]. After washing, all samples (with and without *A. castellanii*) were transferred to Eppendorf tubes with 250–280 mg of glass beads with 1 mm diameter and bead-beaten at 30 shakes per second for 16 cycles of 30 s on and 30 s off to lyse *A. castellanii* trophozoites, pseudocysts and cysts, and release intracellular VIM-PA. A total volume of 800 µL was plated on four separate blood agar plates, resulting in a limit of detection of 0.1 log₁₀ cfu/mL. Serial dilutions of the same sample were also plated on blood agar plates. Blood agar plates were incubated overnight at 37°C, after which the numbers of cfu were counted. From each plate with growth after exposure to disinfectants, one colony was analysed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (Maldi Biotyper, Bruker Microflex LT, Bruker, London, UK) to confirm PA identity. Experiments were performed in at least two independent experiments, each in duplicate wells.

Confocal microscopy

Similar to the in-vitro disinfection, *A. castellanii* trophozoites were grown in the presence or absence of VIM-PA for 14 days. The well contents were then sampled and centrifuged at 1000 g, after which 800 µL of the supernatant was discarded. *P. aeruginosa* antibody 95/159 Alexa Fluor 647 (4 µL) (Novus Biologicals, Littleton, CO, USA) was added to the resuspended cell pellet and incubated at room temperature for 30 min, after which the samples were washed three times with 1 mL PBS and fixed in 0.4% formaldehyde (Fresenius Kabi). Samples were examined by a Leica SP5 confocal laser scanning microscope (Leica, Mannheim, Germany). Autofluorescence was measured with the 488 nm laser line of an argon laser emission BP500-550. Alexa 647 dye was measured with 633 nm laser line and a BP650-720 emission filter. Images were made with an HXP PL APO 63.0 oil immersion lens with a 1.4 numerical aperture. Three-dimensional renderings were made with the Amira software package (Thermo Fisher, Waltham, MA, USA) after deconvolution with the Huygens software (SVI, Hilversum, The Netherlands).

Statistics

GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used to analyse the results. A one-tailed unequal variances *t*-test was used to determine statistically significant difference.

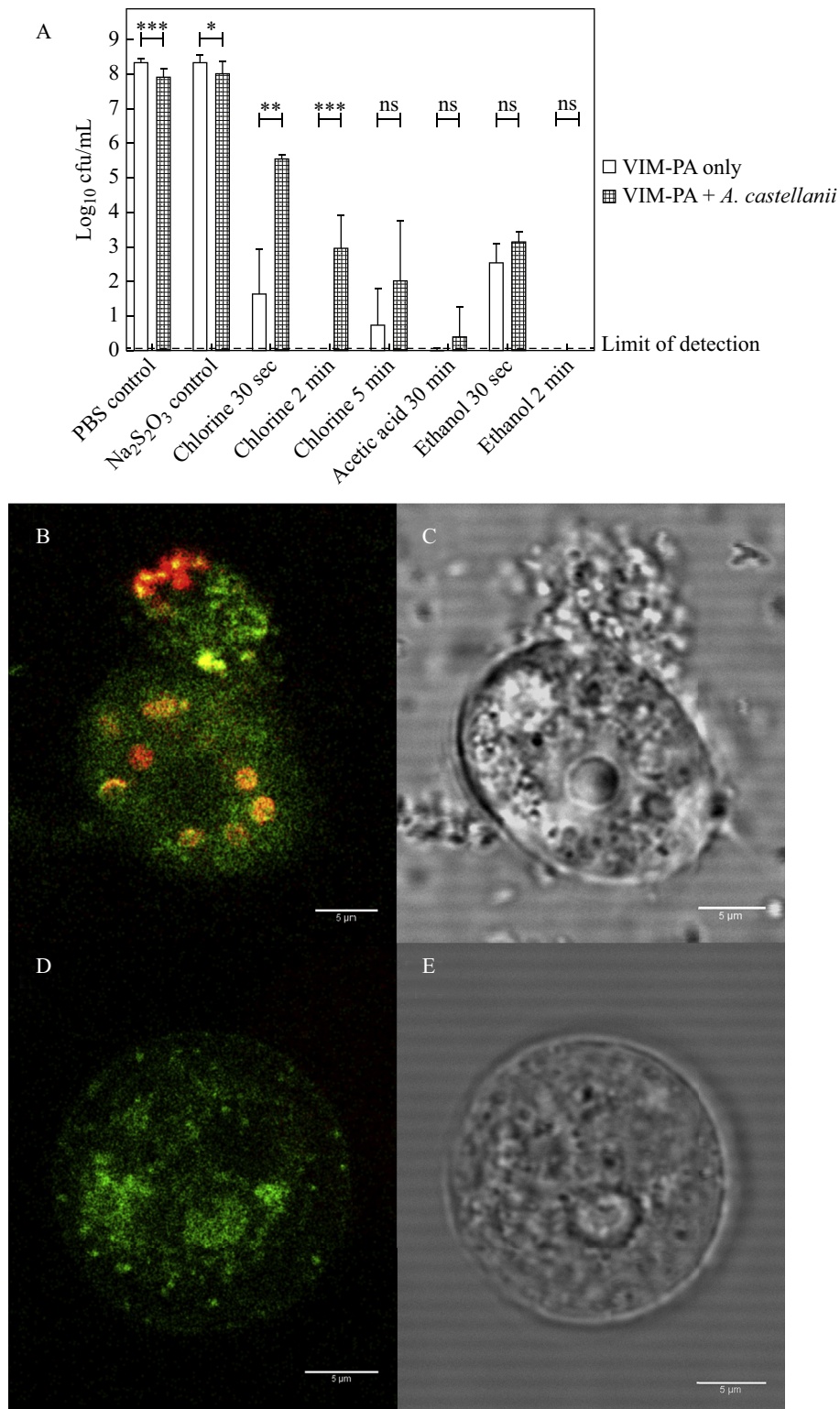


Figure 1. Interaction between *Acanthamoeba castellanii* and Verona-Integron-encoded-Metallo- β -lactamase-positive *Pseudomonas aeruginosa* (VIM-PA). (A) Survival of VIM-PA after disinfection with indicated disinfection methods. Significant differences: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant. (B–E) Confocal microscopy images of *A. castellanii* pseudocysts after 14 days of culture with VIM-PA (B and C) and without VIM-PA (D and E), stained with *P. aeruginosa*-specific antibody (red). Green colour is autofluorescence of *A. castellanii* and VIM-PA.

Results and discussion

This study developed an in-vitro disinfection model to determine the efficacy of VIM-PA disinfection in the presence or absence of *A. castellanii*. In our model, VIM-PA was incubated in PBS with or without *A. castellanii* trophozoites for 14 days preceding in-vitro disinfection in order to allow phagocytosis of the bacteria by *A. castellanii* trophozoites. Survival of VIM-PA after in-vitro disinfection with selected disinfectants for several exposure times is shown in Figure 1. Survival of VIM-PA after chlorine exposure was higher in the presence of *A. castellanii* at all examined time-points. The largest significant difference ($P < 0.001$) was observed after 2 min of chlorine exposure, as all VIM-PA incubated without amoebae were killed, whereas 3 log₁₀ cfu/mL of VIM-PA survived chlorine treatment in the presence of *A. castellanii*. After 5 min of chlorine exposure in the absence of *A. castellanii*, a very small amount of VIM-PA survived (on average 43 cfu/mL), which is a 99.99% reduction compared to a 100% reduction after 2 min of chlorine exposure. In the presence of *A. castellanii*, a time-dependent effect was observed, as survival of VIM-PA decreased upon prolonged exposure to chlorine. By contrast with chlorine disinfection, no significant difference in survival was observed between VIM-PA incubated in the presence or absence of *A. castellanii* after exposure to 24% acetic acid or 70% ethanol. Exposure to 24% acetic acid for 30 min resulted in survival of <1 log₁₀ cfu/mL VIM-PA irrespective of the presence of *A. castellanii*. A 30 s exposure to 70% ethanol resulted in the survival of ~3 log₁₀ cfu/mL of VIM-PA in the absence of *A. castellanii*. A small, non-significant increase in survival of VIM-PA was observed in the presence of *A. castellanii*. Exposure to 70% ethanol for 2 min did result in total eradication of VIM-PA in the sample, irrespective of the presence of *A. castellanii*.

The disinfection model was set up to mimic a hospital water system with the possibility of forming a biofilm. A characteristic of our model is that the microbial biofilm was disrupted before disinfection, as micro-organisms were harvested by vigorous pipetting, resulting in dispersed cells being exposed to disinfectant. We hypothesized that VIM-PA was protected against chlorine disinfection after internalization by *A. castellanii* in our co-culture conditions, which was demonstrated by confocal microscopy using *P. aeruginosa*-specific antibody staining (Figure 1B–E). Surface rendering images can be viewed in Supplementary Videos S1 and S2, which clearly demonstrate the intracellular presence of VIM-PA in *A. castellanii*. We studied only the ATCC *Acanthamoeba* 'Neff' laboratory strain, although it is known that resistance to disinfection may differ between *Acanthamoeba* strains [9]. However, as it has been shown that environmental strains are more resistant to disinfection than laboratory strains, this could mean that the effects we observed are an underestimation of what actually occurs in the environment [9].

The 14-day incubation in our experiments resulted in a mixed population of trophozoites, cysts, and pseudocysts. Pseudocyst formation, like cyst formation, is induced by stress, but pseudocysts are morphologically different from cysts [12]. We observed pseudocyst formation immediately after *A. castellanii* trophozoites were treated with chlorine, contrary to treatment with ethanol (70%) or acetic acid (24%) (Supplementary Videos S3–5). We suggest that these

pseudocyst and cyst stages of *A. castellanii* are responsible for the observed survival of VIM-PA, as our confocal images show the presence of VIM-PA inside pseudocysts (Figure 1B–E). Previous studies have shown that *Acanthamoeba* cysts can survive chlorine exposure, which means that chlorine does not reach intracellular VIM-PA [9].

Current literature points to biofilm formation as the main cause of inefficient bacterial disinfection [13]. In addition to biofilm formation, we now propose another mechanism for inefficient disinfection: survival within *A. castellanii*. Intracellular survival of VIM-PA in *A. castellanii* can be considered synergistic to biofilm formation in the ability to decrease disinfectant efficacy, as *A. castellanii* is known to inhabit biofilms as well [14]. Previous studies have shown that PA transmission may occur from the hospital environment to patients, in whom it can cause serious infections [15]. Our findings indicate that *A. castellanii* can play an important role in the persistence of VIM-PA in hospitals and thus could result in the presence of an environmental source from which VIM-PA transmission to patients can occur.

Other bacteria could take advantage of the protective cover of acanthamoeba as well, as it has been shown that many different types of bacteria can survive inside acanthamoeba. A clear example is *Legionella pneumophila*, as a recent article showed persistent *L. pneumophila* presence in a water system in which acanthamoeba was also present during disinfection with monochloramine [16]. In addition, multiple non-tuberculous mycobacteria have been shown to co-occur with acanthamoeba in a hospital water network [17]. Furthermore, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* have been isolated from *Acanthamoeba* spp. [18]. All these pathogenic bacteria may survive chlorine disinfection in hospital water systems inside amoebae as well.

Our results show that *A. castellanii* can decrease the effectiveness of standard chlorine disinfection of VIM-PA, but not of disinfection with 70% ethanol and 24% acetic acid. As ethanol has a limited applicability for the disinfection of wastewater drains, 24% acetic acid for 30 min is a promising option to obtain effective disinfection. Our findings are in concordance with the results of a recent study which showed that 30 min exposure to 24% acetic acid effectively decontaminated sinks with metallo-β-lactamase-producing PA, and reinforce the use of 24% acetic acid as an environmental decontamination method in clinical practice [10].

Conflict of interest statement

None declared.

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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.2020.09.019>.

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