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Vermamoeba vermiformis resides in water-based heater–cooler units and can enhance *Mycobacterium chimaera* survival after chlorine exposure

M.J. Sarink [a], W.A. van Cappellen [b], A.G.M. Tielens [a], A. van Dijk [c], A.J.J.C. Bogers [c], J.E.M. de Steenwinkel [a], M.C. Vos [a], J.A. Severin [a], J.J. van Hellemond [a,*]

[a] *Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center Rotterdam, The Netherlands*
[b] *Erasmus Optical Imaging Center and Department of Pathology, Erasmus MC University Medical Center Rotterdam, The Netherlands*
[c] *Department of Cardiothoracic Surgery, Erasmus MC University Medical Center Rotterdam, The Netherlands*

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SUMMARY

Background: *Mycobacterium chimaera* colonizes water-based heater–cooler units (HCUs), from which it can spread to patients during surgery. *Vermamoeba vermiformis* is a free-living waterborne amoeba, which was consistently present within HCUs.
Aim: To determine whether these amoebae can be involved in the persistent presence of *M. chimaera*.
Methods: An in-vitro disinfection model.
Findings: Increased survival of *M. chimaera* was observed after chlorine exposure in the presence of *V. vermiformis*. Confocal microscopy demonstrated the intracellular presence of *M. chimaera* in *V. vermiformis*.
Conclusion: In this way, *V. vermiformis* can contribute to the persistent presence of *M. chimaera* in HCUs. Cleaning and disinfection protocols should take this phenomenon into account.

Introduction

Mycobacterium chimaera is a non-tuberculous mycobacterium (NTM) which is ubiquitously found in the aqueous environment [1]. It was first identified in 2004 but was rediscovered as a pathogen in 2013 when Achermann *et al.*

* Corresponding author. Address: Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. Tel.: +31 10 7033510; fax: +31 10 703 38 75.
E-mail address: j.vanhellemond@erasmusmc.nl (J.J. van Hellemond).

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described the first cases of *M. chimaera* infections following cardiothoracic surgery [2]. Since then, many more cases have been identified worldwide. A link was found between disseminated disease caused by *M. chimaera* and the use of water-based heater—cooler units (HCUs) during surgery [1,3]. These HCUs, also known as heater—cooler devices (HCDs), consist of a closed circuit with water tanks and use water as a heat-transfer medium to regulate the body temperature of the patient. In these HCUs, *M. chimaera* and other NTM can be present [4]. Due to the presence of fans and the bioaerosol-forming capacity of *M. chimaera*, transmission of this mycobacterium from the HCU to the surgical field can occur, with subsequent infection of the patient. In order to minimize the risk of infection, HCUs have been modified to reduce aerosolization and compliance with the manufacturer's instructions for cleaning, disinfection and maintenance is advised by regulatory authorities. Several disinfectants are recommended by the manufacturers, including peracetic acid and chlorine (in fact hypochlorite). However, NTM colonization of the HCUs can persist even when these measures are strictly followed, which is thought to be due to the formation of biofilms [4,5]. To prevent infections of the patients, the HCUs used in cardiothoracic surgery in our hospital were placed outside the operating theatre, in a dedicated space, although this solution requires extra tubing and renovation of the operating theatre.

Vermamoeba vermiformis is a water-borne free-living amoeba, which is ubiquitously present in the environment. Worldwide, *V. vermiformis* has been isolated from natural freshwater reservoirs, tap water, swimming pools and hospital environments. The amoeba itself is of very low pathogenicity, although many interactions have been described between *V. vermiformis* and a wide range of pathogenic bacteria [6]. It is known that *V. vermiformis* can act as a host or transport vehicle for *Legionella pneumophila*, *Pseudomonas aeruginosa* and several NTMs. Furthermore, it has been shown that *V. vermiformis* cysts are resistant to chlorine disinfection [7]. We hypothesized that when *V. vermiformis* colonizes HCUs, *M. chimaera* could be present inside these amoebae, thereby resisting disinfectants.

Methods

Setting and sampling

During this study, our hospital used four 3T HCU systems (LivaNova, London, UK). The water within the compartments was replaced with filter-sterilized water each day after use. In addition, the units were cleaned once every two weeks with Puristeril340 (a disinfectant based on peracetic acid). Sampling of HCU-water occurred at regular intervals (see Supplementary Figure S1). Before sampling, the tubing connecting the HCU to the cardiopulmonary equipment was removed and the exit point was cleaned with ethanol 70% after which the contents of each compartment were collected separately in sterile containers.

Strains and materials

The presence of *M. chimaera* in the HCU systems was determined by filtering 200 mL of HCU-water through a 0.2-μm filter. Subsequently, this filter was segregated with a gentleMACS dissociator (Milteny Biotec BV, Leiden, The Netherlands), after which the contents were added to a mycobacterial growth indicator tube (MGIT; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated for six weeks at 35 °C in the BD Bactec MGIT 960 automated mycobacterial detection system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Once growth was detected, DNA was isolated from the culture by MagNa Pure 96 (Roche, Basel, Switzerland) according to the manufacturers' protocol. NTM identity was determined with the GenoType Mycobacterium CM (Hain Lifescience, Nehren, Germany) test system. If *Mycobacterium intracellulare* identity was demonstrated, high-resolution molecular identification was performed by amplification of the 16S—23S rRNA internal transcribed spacer gene by PCR, after which the sequence of the DNA amplification product was determined by Sanger sequencing (Baseclear, Leiden, The Netherlands).

M. chimaera stocks were prepared by addition of 200 μL from an MGIT in which growth was detected by the above-described procedure, to a flask with Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA). Subsequently, this flask was incubated under shaking conditions at 96 rpm at 37 °C until the culture became turbid, after which frozen stocks were prepared by transfer of the flask contents into Eppendorf tubes and stored at −80 °C.

The presence of free-living amoebae in the system 3T HCU was determined by filtering 500 mL HCU-water (sampled as described above) through a 0.2-μm filter, after which the filter was divided into four equal parts and placed upside down on non-nutrient (NN) agar plates seeded with *E. coli* ATCC 25922. Plates were sealed and incubated at 25 °C, after which amoeba presence was checked by twice-weekly microscopic inspection. Morphologically, only one species of amoeba was identified. The amoeba strain used in the disinfection experiments was obtained by subculturing an agar block on new NN agar plates seeded with *Escherichia coli*, as attempts to culture the amoebae in axenic conditions were unsuccessful. The identity of the free-living amoebae was determined by DNA isolation from collected material of a fully grown plate with amoebae, after which the 18S rRNA gene was amplified by PCR using the primers Ami6F1, Ami6F2 and Ami9R, as described previously [8]. The sequence of the amplified DNA product was determined as described above. The amount of free-living amoebae in HCU-water was determined according to Moussa *et al.* [9].

Antibiotic susceptibility testing

Broth microdilution was performed according to ISO standard 20776—1 using ciprofloxacin, doxycycline, moxifloxacin, rifampicin, ethambutol, amikacin and streptomycin, which were all obtained from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). For *E. coli* ATCC 25922, the microdilution plates were examined after overnight incubation at 37 °C. For *M. chimaera*, the microdilution plates were examined after four days of incubation at 37 °C with 5% CO_2.

Disinfection

We used the in-vitro disinfection protocol with chlorine (hypochlorous acid) as described earlier [10] to investigate

whether, in the presence of *V. vermiformis*, *M. chimaera* is protected against disinfectant. This procedure was necessary because the Puristeril340, used for disinfection of the HCUs, cannot be neutralized effectively, which is necessary to investigate the survival of *M. chimaera*. Briefly, *V. vermiformis* trophozoites were collected by adding 1 mL of sterile filtered HCU-water to an NN agar plate seeded with *E. coli* ATCC 25922 and *V. vermiformis*, after which the plate was scraped and liquid was collected. Co-culture experiments were prepared in sterile HCU-water in 24-well plates by addition of 10^7 colony-forming units (cfu) of *M. chimaera* from a frozen stock with or without 10^5 *V. vermiformis* trophozoites in a total volume of 1 mL, corresponding to a multiplicity of infection (MOI) of 100:1. To all wells, ciprofloxacin was added to obtain a 0.5 mg/L concentration in order to kill *E. coli* ATCC 25922 without affecting the growth of *M. chimaera* (see Supplementary Table S1 for antibiotic susceptibility profiles). After four days of incubation at 25 °C, the 24-well plates were placed on ice for 20 min, after which the well contents were collected by vigorous pipetting. The contents of the wells with or without *V. vermiformis* were pooled in two separate flasks and then divided in 1-mL samples over Eppendorf tubes. These tubes were centrifuged at 1000 *g* for 5 min, and the supernatant was discarded. The pellets were resuspended in 1 mL of 1000 ppm available chlorine by immediately and intermittently vortexing to ensure that dispersed cells were all exposed to the disinfectant. Exposure to chlorine was neutralized after 30 s, 2 min, or 5 min by addition of sodium thiosulfate (Sigma, St Louis, MO, USA) to obtain a final concentration of 4 mg/mL sodium thiosulfate. After washing with PBS, all 1-mL samples were transferred to Eppendorf tubes containing 250–280 mg of glass beads of 1 mm diameter. Subsequently, all tubes were bead-beaten at 30 shakes per second for 16 cycles of 30 s on and 30 s off to lyse *V. vermiformis* and to release intracellular *M. chimaera*. Bead-beating did not have negative effects on *M. chimaera* (results not shown). Ciprofloxacin was added to the samples to obtain a final concentration of 0.25 mg/L, after which serial dilutions of the samples were prepared in PBS with 0.25 mg/L ciprofloxacin to prevent growth of *E. coli* ATCC 25922. A total volume of 800 μL (four times 200 μL) of undiluted sample was plated on four separate Middlebrook 7H10 (Difco Laboratories, Detroit, MI, USA) agar plates supplemented with 10% OADC (Becton, Dickinson and Company, Sparks, MD, USA), resulting in a limit of detection of 0.1 log cfu/mL. Ten-fold serial dilutions of the same sample were also plated on Middlebrook plates. All plates were incubated for three weeks at 37 °C with 5% CO_2, after which cfu were counted. From each condition with growth after exposure to disinfectant, three colonies were analysed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (Maldi Biotyper, Bruker Microflex LT, Bruker, London, UK) to confirm *M. chimaera* identity. These disinfection studies were performed in two independent experiments, each in duplicate wells.

Confocal microscopy

V. vermiformis trophozoites were grown on coverslips in the presence or absence of *M. chimaera* for 4 h in a six-well plate, after which the coverslips were fixed with 4% formaldehyde for 1 h. Subsequently, the coverslips were washed twice with sterile filtered HCU-water before permeabilization for 20 min in 0.1% v/v Triton X-100, which was afterwards washed away twice. Samples were then stained using the BD BBL TB Auramine-Rhodamine T staining (Becton, Dickinson and Company, MD, USA) and subsequent propidium iodide staining (0.33 μg/mL in saline-sodium citrate buffer for 5 min) with washing in between with sterile HCU-water. Samples were examined by a Leica SP5 confocal laser scanning microscope (Leica, Mannheim, Germany). Auramine dye was measured with the 458-nm laser line of an argon laser emission BP 470–530. Propidium iodide dye was measured with a 561-nm laser line and a BP 570–640 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).

Results and discussion

In our hospital, the presence of amoebae and *M. chimaera* in the system in the four 3T HCUs was determined at regular intervals over the course of 2 years. The presence of *M. chimaera* over time in the HCUs is shown in Supplementary Figure S1. Amoebae were detected at all nine time points in 18/18 tested samples (100%) and *M. chimaera* was present in 11/102 samples (11%) collected at 13 time points over a period of two years. These results show that despite regular cleaning and disinfection of the HCUs with Puristeril340 according to the manufacturer's instructions, not only *M. chimaera* and other NTM persistently colonize the HCUs, but amoebae do so as well. The concentration of amoebae in HCU-water was determined on two separate occasions from the patient and cardioplegic compartments of an HCU and ranged from 275 to 1204 amoebae per litre. Samples of the content of the HCUs were collected without disturbing any biofilms that might have been present, and therefore, the presence of only planktonic microorganisms was determined. As most amoebae and *M. chimaera* live in biofilms, the actual presence of amoebae and *M. chimaera* in HCUs is probably substantially higher.

Species determination of the isolated amoebae from the HCUs demonstrated their identity as *V. vermiformis*. Clearly, *M. chimaera* as well as *V. vermiformis* are present in the HCUs. Therefore, an in-vitro disinfection model was used to compare the efficacy of chlorine disinfection of *M. chimaera* in the presence and absence of *V. vermiformis*. After four days of incubation with *M. chimaera*, all *V. vermiformis* were encysted (Supplementary Figure S2A, B). At this time point, *M. chimaera* survival after chlorine exposure was determined (Figure 1). The survival after chlorine exposure of these encysted *M. chimaera* was significantly higher than the survival in the absence of *V. vermiformis* (Figure 1a). After incubation without *V. vermiformis*, virtually no *M. chimaera* survived 2-min or 5-min exposure to 1000 ppm available chlorine. In the presence of *V. vermiformis*, however, approximately 2 log cfu/mL and 1 log cfu/mL *M. chimaera* were still viable after 2 min and 5 min of chlorine exposure, respectively. This observed difference in sensitivity to chlorine might be explained by two factors. First, it could be due to the baseline difference in the presence of *M. chimaera* after four days of incubation with or without *V. vermiformis*, as *M. chimaera* counts were approximately 2 log cfu/mL in incubations without *V. vermiformis*, and approximately 4 log cfu/mL in the presence of *V. vermiformis*. This indicates that *M. chimaera* survives in nutrient-poor HCU-water better in the presence of *V. vermiformis* than in its absence,

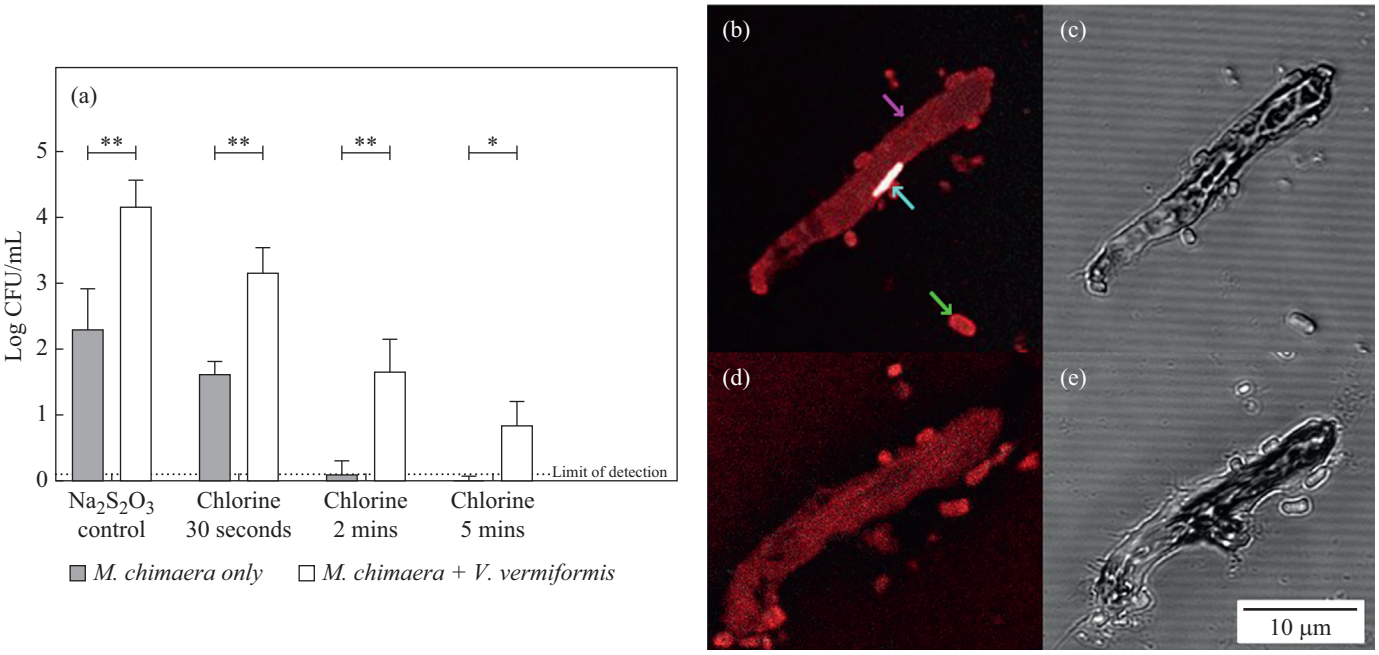

Figure 1. Disinfection and interaction between *Vermamoeba vermiformis* and *Mycobacterium chimaera*, both isolated from heater—cooler units. (a) Survival of *M. chimaera* following chlorine disinfection, after four days of incubation in the presence or absence of *V. vermiformis*. Significant differences: $^{**}P<0.01$; $^{*}P<0.05$. The limit of detection is indicated with the dotted line. Bright-field (c, e) and fluorescence (b, d) confocal microscopy images of *V. vermiformis* incubated for 4 h with *M. chimaera* (b, c) and without *M. chimaera* (d, e). Turquoise arrow indicates *M. chimaera*, pink arrow indicates *V. vermiformis*, green arrow indicates *Escherichia coli*. Samples were permeabilized and stained with propidium iodide, which illuminates *V. vermiformis*, *E. coli* and *M. chimaera* (red), and auramine, which only illuminates *M. chimaera* (white).

thereby resulting in a denser population that is exposed to the effects of chlorine. Second, *V. vermiformis* could also directly protect *M. chimaera* from the effects of chlorine, as it is known that a range of bacterial species can survive within cysts of *V. vermiformis*, among which are mycobacteria [11]. Therefore, we used confocal microscopy to examine the interaction between the *M. chimaera* and *V. vermiformis* strains isolated from the HCU, which revealed *M. chimaera* to be present indeed inside *V. vermiformis* (Figure 1b—e). Surface rendering images can be viewed in Supplementary Videos S1 and S2. Whether *M. chimaera* was located within a vacuole or within the cytoplasm of *V. vermiformis* cannot be determined because this technique does not differentiate between these compartments. However, irrespective of whether *M. chimaera* is present in the vacuole or in the cytoplasm of V. *vermiformis*, *M. chimaera* will be shielded from disinfectants in both situations.

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The formation of a biofilm is known to interfere with disinfection, also in the case of *M. chimaera* in HCUs [4,5,12]. *V. vermiformis* is also an inhabitant of biofilms [13], and as such the amoebae could add to the problem of disinfection. Supplementary Figure S2 shows bright-field images of the situation in our in-vitro co-culture experiments, which might indicate the formation of biofilm, although research into specific evidence of biofilm formation was not conducted. In our in-vitro disinfection model, the biofilm that might have been formed during the four days of incubation was deliberately disrupted during sampling to release amoebae and mycobacteria trapped in biofilms and to expose them to the chlorine. Consequently, our experiments demonstrated enhanced survival of *M. chimaera* by *V. vermiformis* after chlorine disinfection in the absence of biofilms.

M. chimaera persistence in HCUs, despite regular cleaning and disinfection, remains a problem, and therefore, it is now advised to place water-based HCUs outside the operating room. Our study shows that the presence of amoebae, such as *V. vermiformis*, can contribute to the problem of this persistent presence of *M. chimaera* in HCUs. This could either be through increased survival of *M. chimaera* in HCU water which contains *V. vermiformis* (resulting in higher baseline concentrations and subsequent increased survival after disinfectant exposure), or through protection of *M. chimaera* by *V. vermiformis* cysts, as demonstrated by confocal microscopy. Cleaning and disinfection protocols should take this phenomenon of increased survival of *M. chimaera* caused by the presence of amoebae into account.

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Conflict of interest statement

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

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