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Abstract: OBJECTIVE: A growing number of Mycobacterium chimaera infections after cardiosurgery have been reported by several countries. These often fatal infections were traced back to contaminated heater-cooler devices (HCDs), which use water as heat transfer medium. Aerosolization of water contaminated with M. chimaera from HCDs enables airborne transmission to patients undergoing open chest surgery. Infection control teams test HCD water samples for mycobacterial growth to guide preventive measures. The detection limit of M. chimaera in water samples, however, has up to now not been investigated. METHODS: A M. chimaera strain representative of the international cardiosurgery associated M. chimaera outbreak was used to generate a logarithmic dilution series. Two different water volumes, 50ml and 1000ml, were inoculated and after identical processing (centrifugation, decantation, and decontamination) seeded on Mycobacteria growth indicator tube (MGIT) and Middlebrook 7H11 solid media. RESULTS: MGIT consistently showed a lower detection limit as 7H11 solid media, corresponding to a detection limit of a concentration of $1.44\times10^4$ CFU/ml for 50ml and 2.4 CFU/ml for 1000ml water samples. Solid media failed to detect M. chimaera in 50ml water samples. CONCLUSION: Depending on water volume and culture method, major differences exist in the detection limit of M. chimaera. In terms of sensitivity, 1000ml water samples in MGIT media performed best. Our results have important implications for infection prevention and control strategies in mitigation of the M. chimaera outbreak and healthcare water safety in general.

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Detection limit of *Mycobacterium chimaera* in water samples for monitoring medical device safety: insights from a pilot experimental series

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Title: Detection limit of Mycobacterium chimaera in water samples for monitoring medical device safety: insights from a pilot experimental series

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Abbreviated title: detection limit of M. chimaera in water samples

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Objective: A growing number of *Mycobacterium chimaera* infections after cardiosurgery have been reported by several countries. These often fatal infections were traced back to contaminated heater-cooler devices (HCDs), which use water as heat transfer medium. Aerosolization of water contaminated with *M. chimaera* from HCDs enables airborne transmission to patients undergoing open chest surgery. Infection control teams test HCD water samples for mycobacterial growth to guide preventive measures. The detection limit of *M. chimaera* in water samples, however, has up to now not been investigated.

Methods: A *M. chimaera* strain representative of the international cardiosurgery associated *M. chimaera* outbreak was used to generate a logarithmic dilution series. Two different water volumes, 50ml and 1000ml, were inoculated and after identical processing (centrifugation, decantation, and decontamination) seeded on Mycobacteria growth indicator tube (MGIT) and Middlebrook 7H11 solid media.

Results: MGIT consistently showed a lower detection limit as 7H11 solid media, corresponding to a detection limit of a concentration of ≥1.44x10^4 CFU/ml for 50ml and ≥2.4 CFU/ml for 1000ml water samples. Solid media failed to detect *M. chimaera* in 50ml water samples.

Conclusion: Depending on water volume and culture method, major differences exist in the detection limit of *M. chimaera*. In terms of sensitivity, 1000ml water samples in MGIT media performed best. Our results have important implications for infection prevention and control strategies in mitigation of the *M. chimaera* outbreak and healthcare water safety in general.
Introduction

*Mycobacterium chimaera*, a member of the *Mycobacterium avium* complex first described in 2004 [1], recently stirred the awareness of hospital epidemiologists and infectious disease specialists due to its association with infections following cardiosurgery. Since the description of the first two cases in 2013 [2], an increasing number of *M. chimaera* infections associated with cardiosurgery interventions has been reported from numerous countries [3-8]. *M. chimaera*, other NTM as well as other bacteria have been isolated from the water system of heater-cooler devices (HCDs) [9-12]. Also, air samples collected next to operating HCDs that were contaminated with *M. chimaera* grew *M. chimaera* [13, 14]. Aerosols containing *M. chimaera* are being generated in the water reservoir of HCDs and subsequently dispersed by a fan used for heat exchange [13]. Airborne transmission from contaminated HCDs to implants and the surgical site during cardiac surgery is considered the most likely route of infection [15]. Endovascular or disseminated infections caused by *M. chimaera* are characterized by often delayed diagnosis and poor prognosis [16]. Despite aggressive antibiotic therapy and revision surgery, curability remains uncertain, as relapses may occur even after prolonged antimicrobial therapy [16, 17] (personal communication Barbara Hasse, Zurich, Switzerland).

Given the clinical relevance, surveillance cultures from HCD water samples are often employed to assess efficacy of decontamination procedures [10, 13, 18]. However, no data have been published on the detection threshold of *M. chimaera* in water samples. We sought to close this gap with laboratory-based serial dilution tests.
Methods

Stock solution, dilution series 1 to 8

The isolate chosen for this investigation was *M. chimaera* ZUERICH-1 (DSM 101591). The genome of the strain has been sequenced completely (NCBI GenBank accession NZ_CP015272) and is characterized by a median pairwise distance of only 12 single nucleotide polymorphisms in comparison to isolates gathered from LivaNova (London, UK; formerly Sorin or Stöckert) heater cooler units; thus is highly related to the strains recovered from HCDs and infected patients [12]. After five days of incubation in a MGIT tube (MGIT 960; Becton Dickinson, Sparks, MD, USA) a suspension of 0.5 McFarland, corresponding to $10^7$ CFU/ml [19], was generated in 5ml sterile water. Five-day growth cultures were chosen to gain mycobacteria whilst showing logarithmic growth behavior. Tube #1 contained the undiluted suspension. Tube #2 to #8 were prefilled with 36ml sterile water each, to create a logarithmic dilution series, i.e. dilution #2 to #8. Dilutions #2 to #8 resulted from adding 4ml of the solution from one tube into the next consecutive tube, i.e. dilution #2 was generated by adding 4ml of solution #1 to 36ml of sterile water in tube #2, the dilution #3 by adding 4ml of dilution #2 to 36ml sterile water in tube #3, etc. To reduce clumping of mycobacteria, the initial solution for each dilution was dispersed by drawing up and expelling 10 times through a 26-gauge needle attached to a 1-ml syringe [20].

To verify the resulting concentrations, 0.1ml of dilutions #4 to #8 were seeded on 7H11 solid media (BD Difco Mycobacteria 7H11 Agar; Becton Dickinson) with 10% OADC (oleic albumin dextrose catalase; Becton Dickinson) in triplicates and incubated at 37 ± 1.5 °C and 5-10% CO$_2$ for seven weeks or until positive. Solid media agar plates were sealed with Parafilm M (Bemis, Neenah, WI, USA) to prevent drying. Concentrations of dilutions #1 to #3 were
calculated based on the results of dilution #4. Due to the logarithmic dilution, multiplication by 10 was necessary to calculate each precedent number of CFUs and resulting concentrations. Cultures were assessed weekly. In case of growth, colonies were counted and identified as mycobacteria by acid-fast staining. Upon confirmation, colonies were suspended in 3ml 0.9% sodium chloride. According to the instructions of the manufacturer, 0.5ml of this suspension underwent molecular identification using InstaGene Matrix (Biorad, Hercules, CA, USA). A modified LightCycler 16S rDNA assay was performed to identify non-tuberculous mycobacteria [21]. If the *Mycobacterium* sp. probe was positive, the LightCycler PCR amplicon was purified and subjected to sequencing. SmartGene IDNS 16S rDNA database (SmartGene Zug, Switzerland) was used for comparisons of 16S rDNA homology enabling species identification.

**Water samples**

**Water samples of 50ml; dilution series #1a to #8a**

For each 50ml water sample, 1ml of dilutions #1 to #8 were added to each 49ml sterile water resulting in the dilutions #1a to #8a. Samples were centrifuged at 3300g for 15 minutes; after centrifugation samples were decanted and resuspended with sterile water until 5ml left-over including pellet remained in the primary tube. The decontamination method for inactivation of non-mycobacterial species in water samples has been evaluated before setting up the currently presented study (Swiss National Center for Mycobacteria, University Zurich, data not shown). Samples have been decontaminated as follows: 5ml decontamination solution (BBL MycoPrep Kit, BD, Franklin Lakes, NJ, USA) was added to the
primary tube. After 15 minutes, 30ml phosphate buffer (BBL MycoPrep Kit, BD, Franklin Lakes, NJ, USA) was added. After decontamination a volume of 500µl was used for MGIT and 100µl were seeded on 7H11 solid media. Dilution #6a to #8a were seeded undiluted; dilution #5a both, undiluted and diluted to 1:10 (using phosphate buffer); dilution #4a diluted both, to 1:10 and 1:100; dilution #3a diluted both, to 1:100 and 1:1,000; dilution #2a both, to 1:1,000 and 1:10,000; and dilution #1a both, to 1:10,000 and 1:100,000. Assessment of solid media for growth and identification was performed as described above for the dilution series #1 to #8.

Water samples of 1000ml; dilution series #1b to #8b

For each 1000ml water sample 20ml of dilutions #1 to #8 were added to each 980ml sterile water resulting in dilutions #1b to #8b. Centrifugation, decantation, resuspension and decontamination procedure were performed as described above for the 50ml water samples. After decontamination, a volume of 500µl was used to inoculate MGIT tubes and 100µl for 7H11 solid media. Dilution #7b to #8b were seeded undiluted; dilution #6b both, undiluted and diluted to 1:10 (using phosphate buffer); dilution #5b diluted both, to 1:10 and 1:100; dilution #4b both, diluted to 1:100 and 1:1,000; dilution #3b diluted both, to 1:1,000 and 1:10,000; dilution #2b diluted both, to 1:10,000 and 1:100,000; and dilution #1b diluted both, to 1:100,000 and 1:1,000,000 diluted. Assessment of solid media for growth and identification was performed as described above for the dilution series #1 to #8.
Calculation of the detection limit

For determination of the detection limit the highest dilution resulting in growth of *M. chimaera* was considered for both volumes and methods, respectively. The concentration responding to the detection limit was calculated by dividing the initial inoculum by the total volume.
Results

Bacterial concentrations in stock solution and dilution series

Dilution #4 through #8 showed a median count of 720, 131, 36, 12 and 21 CFU per 100µl, equaling concentrations of 7200, 1310, 360, 120, and 210 CFU/ml, respectively (Table I). For dilutions #1, #2, and #3, concentrations of $7.2 \times 10^6$ CFU/ml, $7.2 \times 10^5$ CFU/ml and $7.2 \times 10^4$ CFU/ml, respectively, were extrapolated based on the concentration of dilution #4.

Detection of *M. chimaera* in the two investigated water sample volumes

**Water samples of 50ml**

With water samples of 50ml, MGIT detected *M. chimaera* only in the highest two concentrations tested, i.e. dilution #1a and #2a, corresponding to a detection limit of a bacterial concentration of $\geq 1.44 \times 10^4$ CFU/ml (Table II). In dilution #1a (inoculum of $1.44 \times 10^5$ CFU/ml) growth of *M. chimaera* was reported five days earlier than in dilution #2a (inoculum of $1.44 \times 10^4$ CFU/ml). *M. chimaera* did not grow at all on 7H11 solid media in any of the test dilutions.

**Water samples of 1,000ml**

When water samples of 1000ml were used, MGIT was able to identify *M. chimaera* in all tested dilutions, equaling a detection limit of a bacterial concentration of $\geq 2.4$ CFU/ml (Table III). Comparing the proportion of positive culture results in MGIT between 50ml and 1000ml water samples, resulted in a significant difference (Fisher’s Exact test, $p=0.007$). The 7H11 solid media detected *M. chimaera* in dilution #1b (inoculum of $1.44 \times 10^5$ CFU/ml; 2 colonies) and dilution #5b (inoculum of 26.2 CFU/ml, 1 colony), but not in dilutions #2b to #4b.
Discussion

By comparing 7H11 and MGIT media for mycobacterial culture and comparing two different water sample volumes of 50 and 1000ml, we gathered relevant insights on the detection limit of *M. chimaera* in water. The sensitivity of MGIT for detecting *M. chimaera* in centrifuged 1000ml water samples was excellent in contrast to centrifuged 50ml water samples with a detection limit of over 10,000 CFU/ml.

The use of 7H11 solid media proved unsuitable for *M. chimaera* detection for either of the two sampling volumes. Nevertheless, due to its broader growth conditions 7H11 solid media offer the advantage of detecting other bacterial species, which have been also reported in HCDs [22]. In the 1000ml dilution series growth of *M. chimaera* on 7H11 solid media was inconsistent. This observation might be explained by a stochastic phenomenon. Clump formation or cording of *M. chimaera* in the dilutions could have hindered adequate inoculation of 7H11 solid media. However, we cannot prove this hypothesis, as we didn’t perform microscopy in these samples. For the present study, we decided to use 7H11 media sealed with Parafilm M and not Lowenstein-Jensen tubes as solid media. 7H11 media allow to recover mycobacteria more easily and previous studies indicated non-inferior recovery rates for NTMs, if desiccation of the media was prevented by Parafilm M [23]. In terms of sensitivity, 1000ml water sampling combined with MGIT represents the method of choice to detect *M. chimaera* from environmental water sources such as water-bearing medical devices including HCDs, extracorporeal circuit membrane oxygenators (ECMOs) as well as water fountains, and tap water. One barrier in implementation may be the need for large volume centrifuges. However, if water samples are tested to assist in risk management
decision, the significant increased sensitivity of 1000ml samples should promote motivation for acquisition of corresponding equipment. If the water volume in the source to be tested is less than 1000ml, 50ml water samples and MGIT media can be employed, while recognizing the considerably lower sensitivity of this approach. If sample volumes between 50ml and 1000ml were used, feasibility would be even more complex, as pooling and multiple centrifuge runs would be required. According to a recent report, it took six months until *M. chimaera* could be isolated from factory-new HCDs that were most likely already contaminated during their production [10]. Hypothetically, this observation might be due to the fact that a certain timespan was needed for mycobacterial growth until the detection limit of 50ml samples was reached.

A single study tested *M. chimaera* contamination of HCDs using swabs from surfaces and tubings of ECMOs [24]. Sampling of inner surfaces of tubes or tanks could be taken into consideration as an alternative method to assess the contamination status of HCDs. However, gaining representative samples requires partial disassembly of HCDs thus complicating regular testing. There are currently no data on the relative sensitivity comparing the two methods.

Routine surveillance cultures of HCD water for mycobacteria were discussed controversially with the main argument being the undefined negative predictive value due to the unknown limit of detection [25, 26]. With respect to feasibility, Total Viable Counts (TVCs) were frequently used to assess contamination of HCDs in general. In a recent study with repeated
determination of TVCs and selected samples for mycobacterial cultures, presence of *M. chimaera* was only described, if TVDs were high [27]. With respect to the co-existence of *M. chimaera* and other pathogens in biofilms, a positive correlation between TVCs and presence of *M. chimaera* could be hypothesized, but up to now insufficient data exist to support this hypothesis. Notably, in this study water samples of 100ml were used which are likely inferior to 1000ml samples in terms of sensitivity. Awareness of the detection limit of *M. chimaera* in HCD water samples is relevant for the mitigation of the current outbreak associated with cardiac surgery [14]. An early and reliable detection of NTM contamination of water-containing medical devices is pivotal to develop a safe technical solution and assists in an effective management of these devices in clinical use. Our results should also be considered in guidelines for HCD management.

In environmental microbiology, limit of detection assays have been mainly established for *Legionella* spp. [28]. For mycobacterial detection in environmental samples, only relatively small studies showing widely varying results have been reported, e.g. few studies demonstrated the occurrence of opportunistic NTM species in environmental water samples [29-31]. A study by Radomski et al. investigated the effect of different decontamination methods and culture media supplementation with antibiotics in the detection of NTM from environmental samples [32].

A major strength of our study is the use of the purposefully selected *M. chimaera* strain. Given its proven clinical relevance a *M. chimaera* strain from a patient infected during
cardiosurgery in the context of the current global outbreak was chosen. Strikingly, in a recent study using whole genome sequencing this strain clustered with all isolates except one gathered from individuals suffering from HCD-associated *M. chimaera* infection, the vast majority of *M. chimaera* isolates from LivaNova HCDs in use and an isolate derived from the LivaNova manufacturing site [12].

This study has limitations. Our experiments focused on a single, but in the context representative, *M. chimaera* strain. With respect to the limited number of experiments, our results are of pilot character. The concentration of *M. chimaera* in HCD water systems that is associated with an infectious risk for patients is unknown. This risk, however, certainly depends on multiple additional factors beyond bacterial concentration in HCD water, including HCD design features, HCD positioning and orientation, operating room ventilation profile, and *M. chimaera* strain properties. Notably, besides *M. chimaera* other NTM such as *M. abscessus* have been associated with HCD derived infections [11]. Similar experiments with other NTM are lacking, but in a study on HCD surveillance we were able to culture several NTM species with using the described methods in 50ml water samples [10]. Until sounder evidence becomes available, any contamination of HCD water with *M. chimaera* and other NTM should trigger preventive measures to guarantee patient safety [11, 25]. Decontamination procedures are complicated by biofilm formation within HCDs enabling persistence of *M. chimaera* [17, 18]. Furthermore, air culture sensitivity for NTM remains unknown despite its central importance for HCD safety. Studying microbial detection limits in air volumes is notoriously challenging [33, 34] and was beyond the scope of the current protocol.
Conclusion

Our study proved major differences in the detection limit for *M. chimaera* for the assessed laboratory protocols. Highest sensitivity was achieved by 1000ml water samples in MGIT media and solid media failed to detect *M. chimaera* in 50ml water samples. These findings have important implications for infection prevention and control strategies regarding the infectious risk associated with NTM and stagnant water in healthcare settings. Current international recommendations on the management of HCD may have to be adapted accordingly.
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Associated With Heater-Cooler Devices: Piecing the Puzzle Together. *Infect Control


**Table III. Culture results for dilution series of 1000ml water samples**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1b</th>
<th>2b</th>
<th>3b</th>
<th>4b</th>
<th>5b</th>
<th>6b</th>
<th>7b</th>
<th>8b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum (CFUs)</td>
<td>1.44x10^8</td>
<td>1.44x10^7</td>
<td>1,440,000</td>
<td>144,000</td>
<td>26,200</td>
<td>7200</td>
<td>2400</td>
<td>4200</td>
</tr>
<tr>
<td>MGIT (time until positive, days)</td>
<td>Pos (5)</td>
<td>Pos (7)</td>
<td>Pos (9)</td>
<td>Pos (15)</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>7H11 (CFU counted)</td>
<td>2 col.*</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1 col.*</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>7H11 (CFU/ml)¶</td>
<td>2x10^6</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1x10^7</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

The dilutions were plated once.

Note, a negative control showed no growth in MGIT liquid media and on 7H11 solid media.

CFU, colony forming unit; MGIT, mycobacterial growth indicator tube.

* seeded 1:100 000 diluted

# seeded 1:10 diluted

¶ calculated based on CFU count on 7H11
Table II. Culture results for dilution series of 50ml water samples

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1a</th>
<th>2a</th>
<th>3a</th>
<th>4a</th>
<th>5a</th>
<th>6a</th>
<th>7a</th>
<th>8a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum (CFUs)</td>
<td>7,200,000</td>
<td>720,000</td>
<td>72,000</td>
<td>7200</td>
<td>1310</td>
<td>360</td>
<td>120</td>
<td>210</td>
</tr>
<tr>
<td>MGIT (time until positive, days)</td>
<td>Pos (12)</td>
<td>Pos (17)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>7H11 (CFU counted)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>7H11 (CFU/ml)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

All dilutions were plated once.

Note, a negative control showed no growth in MGIT liquid media and on 7H11 solid media.

CFU, colony forming unit; MGIT, mycobacterial growth indicator tube.
Table I. Quantification of stock solution and logarithmic dilution series

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU count 1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>688</td>
<td>115</td>
<td>35</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>CFU count 2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>720</td>
<td>131</td>
<td>36</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>CFU count 3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>792</td>
<td>140</td>
<td>48</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>Median CFU count</td>
<td>720,000*</td>
<td>72,000*</td>
<td>7200*</td>
<td>720</td>
<td>131</td>
<td>36</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Concentration (CFU/ml)</td>
<td>7,200,000</td>
<td>720,000</td>
<td>720,00</td>
<td>7200</td>
<td>1310</td>
<td>360</td>
<td>120</td>
<td>210</td>
</tr>
</tbody>
</table>

Note, a negative control showed no growth in MGIT liquid media and on 7H11 solid media.

CFU count reported for seeding of 100µl of the corresponding dilutions on 7H11 solid media.

* extrapolated from dilution #4

nd, not done.