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Detection of *Mycobacterium chimaera* in medical device water samples by customised real time PCR using a InGenius platform

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***Mycobacterium chimaera*, belonging to the *Mycobacterium avium* complex, is an opportunistic environmental mycobacterium which has been isolated from medical device water samples such as Heater Cooler Units (HCU). Laboratories currently use culture-based diagnostic methods to detect *M. chimaera*, but these take a long time to obtain results. The aim of this study is to test and define the Limit of Detection (LoD) of a Real-Time Polymerase Chain Reaction test (RT-PCR) specific for *M. chimaera*, using a sample-to-result InGenius platform, performed on medical device water samples collected at the referral centre for the detection of mycobacteria from environmental specimens of Bologna, Italy. A total of 285 water samples were included in this study. The sensitivity and specificity of RT-PCR compared to culture were 60.5% and 98.8% respectively, with an overall agreement of 82.1% and a positive predictive value of 97.4%. The LoD calculated was approximately 2900 CFU/ml. In conclusion, this study confirmed that detection of *M. chimaera* with RT-PCR could support culture-based methods in reducing the time necessary to identify highly colonised HCUs, with high positive predictive values. Therefore, we suggest performing this customised RT-PCR on concentrated decontaminated water samples, shutting down and thoroughly disinfecting positive HCUs, to reduce the risk of patient infection.**

Keywords Nontuberculous mycobacteria, *M. chimaera*, Medical device water samples, Heater-cooler units, Real-time PCR, ELITe InGenius® platform

Mycobacterium chimaera, identified and included in the *Mycobacterium avium* complex by Tortoli et al. in 2004[1], is a slow-growing nontuberculous Mycobacterium (NTM) ubiquitously found in the environment, including domestic and hospital water distribution systems[2,3].

M. chimaera is able to colonise medical devices and resist common disinfection methods[4], therefore invasive cases of mycobacterial infection have been associated with aerosols produced by the use of heater-cooler units (HCU) during cardiac surgery[5,6]. To understand the origin of HCU colonisation, WGS and Fourier Transform Infrared Spectroscopy (FTIRS) were used for microbial typing, suggesting that the plumbing system could represent a source of HCU contamination and, potentially, patient infection[7,8].

In order to control the contamination of HCU by *M. chimaera*, an active surveillance programme was encouraged by the European Centre for Disease Prevention and Control (ECDC) in 2015[9]. Laboratories currently use culture-based diagnostic methods to detect *M. chimaera* in environmental specimens (e.g. 1 L of water), but these techniques require a long time to obtain results and confirm colonisation. During this time HCUs under investigation continue to be used during cardiac surgery, with the consequent risk to patients.

The aim of this study was to assess the performance and define the Limit of Detection (LoD) of a target-specific assay for the detection of *M. chimaera* DNA by a Real-Time Polymerase Chain Reaction test (RT-PCR) on a large number of water samples from the Italian Emilia Romagna Region HCU surveillance programme.

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Materials and methods

Study design

This is a retrospective study performed at the Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria of Bologna, as referral centre for the detection of mycobacteria from environmental specimens, including HCU water samples, for the metropolitan area of Bologna. Active surveillance and microbiological investigation are currently ongoing according to the recommendations of the National Health Authorities[10]. From January 2020 to February 2023, 927 water samples were collected from 79 HCUs for the detection of *M. chimaera*. The HCUs were located in the cardiac surgery departments of 5 hospitals in the Emilia Romagna Region. 308 samples came from 17 HCU40 produced by Maquet (Getinge Group, Germany), and 619 samples from 62 Stockert 3T HCUs manufactured by LivaNova (Sorin Group, Germany).

Sample processing

One litre of each HCU water sample was collected from both circuits (patient and cardioplegia) in a sterile plastic bottle containing sodium thiosulphate and processed within 24 h. Samples were concentrated 100 times by filtering through nitrocellulose membranes (Ø 0.45 µm) using Microsart filtration system (Sartorius, Germany) and resuspended in 10 ml of Ringer's solution. Concentrated samples were digested and decontaminated with N-Acetyl-L-Cysteine Sodium Hydroxide Mycoprep solution (Becton Dickinson, USA) and the resulting sediment was resuspended in 2 ml of Ringer's solution. 250 µl of this suspension were inoculated onto a Lowenstein Jensen solid slant medium (Biolife, Italy) and 500 µl into MGIT liquid culture (Becton Dickinson, USA) as per standard procedure according to CLSI guideline[11]. Solid and liquid cultures were considered negative after 42 days of incubation without isolation of any Mycobacteria. 250 µl aliquots of decontaminated sample were stored at -20 °C to be tested by RT-PCR on ELITe InGenius® platform.

Positive cultures were verified for acid-fast bacilli by microscopic examination using Ziehl-Neelsen stain. Time to positivity (TTP) was defined as the number of days from MGIT inoculation to the positive culture result using Epicenter software (Becton Dickinson, USA).

Positive mycobacteria cultures were first identified as NTM by GenoType Mycobacterium CM CE-IVD kit (CM, Bruker, Germany) and then as *M. chimaera* by GenoType NTM-DR CE-IVD kit (NTM-DR, Bruker, Germany). All NTM species were confirmed by MALDI-TOF analysis (Bruker, Germany).

*M. chimaera*RT-PCR on ELITe InGenius® system

A 79 bp sequence in the SR1 conserved region of *M. chimaera* genome, previously described by Zozaya-Valdés et al.[12], was amplified by RT-PCR using customised primers and TaqMan probe (Thermo Fisher Scientific, USA). The Master Mix (MMX) is shown in Table 1.

DNA extraction and amplification were performed on sample-to-result ELITe InGenius® platform (Elitech group, Italy). For DNA extraction, 250 µl of decontaminated water sample were inactivated at 95 °C for 30 min and 2 µl of TaqMan™ Universal DNA Spike-in Control added as internal control and amplified by TaqMan® Gene Expression Assays (Thermo Fisher Scientific, USA). Automatic extraction was performed with ELITe magnetic beads-based cartridges (Elitech group, Italy) and DNA was eluted in 50 µl of ultrapure nuclease-free water. For each sample, 5 µl of eluted DNA were used in the amplification mix to a final volume of 23 µl.

To evaluate any DNA contamination during the process, a negative sample (250 µl of ultrapure water) was added in all the extraction and subsequent amplification sessions.

In order to evaluate the Limit of Detection (LoD) of this custom assay, serial dilutions starting from 1.5×10^8 CFU/ml (0.5 Mc Farland bacterial suspension) to 10^1 CFU/ml were performed in 3 different experiments, each in triplicate, using a reference *M. chimaera* isolate (DSM 44623).

For each dilution, 100 µl were inoculated onto a Middlebrook 7H11solid medium (BD, USA) to count colonies, and simultaneously 100 µl of the same dilution was added to 150 µl of ultrapure water to be used for DNA extraction and RT-PCR amplification, in order to calculate the LoD of the test. LoD was considered the mean number of colony forming units (CFU) per ml, counted in solid media relating to the lowest serial dilution that obtained a positive RT-PCR result.

Statistical analysis

Determination of sensitivity, specificity, positive predictive value, 95% confidence intervals and the agreement between culture and customised *M. chimaera* RT-PCR on ELITe InGenius® platform was determined by two-by-two contingency tables. Significant differences were evaluated by χ^2 test using GraphPad Prism version 8.0.1 (Prism 8, San Diego, CA).

Amplification mix component	Sequence 5'-3'	Final concentration	MMIX volume
TaqMan® Probe	ACTCAAACACCTGACGAGTCA	0.2 µM	2.4 µl
Primer forward	ACTTGACGAGGTCTTGCAGG	0.4 µM	1.2 µl
Primer reverse	GACGGCATAGAGATTCGCCA	0.4 µM	1.2 µl
TaqMan® Fast Advanced MMX		2X	12.0 µl
TaqMan® Gene Exp. Assays		20X	1.2 µl
Total MMX volume			18.0 µl

Table 1. Customised real-time PCR master mix.

Mann Whitney test was used to compare TTP between samples with positive and negative RT-PCR results. Statistical significance was set at $P < 0.05$.

Results

One hundred and twenty-four (13.4%) of 927 water samples were culture-positive for *M. chimaera* (20 samples came from 8 of the 17 Maquet devices, and 104 from 52 of the 62 LivaNova devices). 1 sample was culture-positive for *M. chelonae*, 19 for *M. gordonae* and 21 for *M. paragordonae*. 762 (82.2%) HCU water samples were NTM culture-negative.

For this study we evaluated the performance of customised *M. chimaera* RT-PCR on ELITe InGenius® platform from 285 decontaminated water samples. *M.chimaera* culture-positive samples were analysed to evaluate the sensitivity of the test. 161 water samples were culture-negative for *M. chimaera*, including 41 water samples which tested positive for other NTM. These were analysed to assess specificity.

Twenty-one (7%) of the 285 tests performed by RT-PCR were invalid, due to Internal Control failure (incorrect extraction or inhibitor carry-over). All samples with an invalid result were re-tested and a valid result was obtained.

75 (60.5%) of the 124 *M. chimaera* culture-positive samples were also positive with RT-PCR, while for 49 samples the target was not detected. 159 (98.8%) of the 161 *M. chimaera* culture-negative samples were confirmed negative by RT-PCR. Therefore, the overall sensitivity and specificity of RT-PCR on decontaminated water samples compared to culture was 60.5% (95% CI: 0.517–0.687) and 98.8% (95% CI: 0.956–0.997), respectively.

The overall agreement between RT-PCR and culture was 82.1% (K = 0.62) and the positive predictive value of the test was 97.4% (95% CI: 0.910–0.995).

In liquid culture, the samples that tested RT-PCR positive had a median time to positivity (TTP).

of 11.7 days, significantly lower than the TTP median of samples that tested RT-PCR negative (15.0 days) ($p = 0.0018$).

Table 2 reports the number of colonies counted in 7H11 solid media and the corresponding Cycle Treshold (CT) results obtained by customised *M. chimaera* RT-PCR in 3 independent experiments, each performed in triplicate, using *M. chimaera* DSM 44623 strain. No RT-PCR sessions were contaminated, as negative controls were *M. chimaera* negative.

The LoD was calculated as the mean CFU obtained in the lowest serial dilution with a positive RT-PCR result (732 ± 33 CFU in 250 µl), multiplied by 4 to convert to CFU/ml.

Therefore, the limit of detection (LoD) of custom *M. chimaera* RT-PCR on the ELITe InGenius® platform was set at 2929 ± 132 CFU/ml of concentrated water sample, with a mean CT of 35 ± 1, leading to detection of *M.chimaera* DNA starting at 29 CFU/ml from water circulating in medical devices.

Discussion

Several worldwide studies have reported that Nontuberculous mycobacteria (NTM) have been isolated from healthcare facility water systems and medical devices that use ultrapure water, leading to an increased risk of patient exposure to these pathogens[3,13–15].

In particular, *M. chimaera*, a slow-growing NTM, has been isolated from contaminated heater-cooler devices (HCU) used in open-heart surgery[5,6,8] and in our previous study we demonstrated that *M. chimaera* subtypes circulating in hospital plumbing systems share a highly similar genetic profile with isolates colonising medical devices[7].

This validation study was carried out on a large number of HCU water samples, longitudinally collected during the period 2020 to 2023 from five Italian hospitals and investigated for the presence of *M. chimaera* according to ECDC protocol[9]. 124 samples out of 927 (13.4%) were culture-positive for *M. chimaera.* 20 of these came from 308 samples (6.5%) collected from HCU40 (Getinge, Germany) and 104 came from 619 samples (16.8%) collected from Stockert 3T models (LivaNova, Germany), confirming that *M. chimaera* is still circulating despite the disinfection procedure recommended by the manufactures, and that the LivaNova model was the most frequently contaminated, as already described by Ditommaso et al.[16].

This is the first evaluation study performed on sample-to-results ELITe InGenius® platform (ELITe) for the detection of *M. chimaera* genome using a customised TaqMan RT-PCR based on sequences previously described by Zozaya-Valdés et al.[12]. This automated system simplifies the laboratory workflow by integrating nucleic acid extraction and purification, real-time PCR amplification, detection of the target sequence with melt-curve analysis and result interpretation with a faster turnaround time[17], compared to other available RT-PCR for *M.chimaera*, which require more complex manual skills[16,18].

The overall RT-PCR sensitivity compared to culture, obtained on a large number of *M .chimaera* culture-positive water samples tested in this study ($n = 124$), was 60.5%, whereas in the pilot study performed by Zozaya-Valdés, with 100% sensitivity, the number of water samples analysed was definitely lower ($n = 33$)[12].

Here, the sensitivity was higher compared to a similar study performed with a propidium monoazide (PMA)-PCR assay (that amplifies 16 S rRNA to detect the NTM genome) on HCU water samples with a sensitivity of 38.9% compared to culture[19].

However, samples with a low microbial load failed to be detected with this molecular method. In fact, the mean times to positivity (TTP) in liquid culture of samples that tested RT-PCR positive were significantly lower than samples that tested RT-PCR negative ($p < 0.0001$) .

The Limit of detection (LoD) of the customised *M. chimaera* RT-PCR on ELITe InGenius® platform was approximately 2900 CFU/ml, in line with other molecular kits for the detection of bacterial pathogens, such as *Legionella pneumophila* and *Chlamydophila pneumoniae* (1250 and 5000 copies/ml, respectively) on the same platform (Respiratory Bacterial ELITe MGB® Panel, ELITechGroup, Italy). However, the LoD was higher than

Replicate	CFU of *M. chimaera*	CT of *M. chimaera*	CT of internal control
1	7000#	30.01	23.9
	7200#	29.82	24.0
	7700#	29.74	24.1
	705	34.07	24.1
	723	34.51	24.0
	769	36.16	24.4
	69	NA	24.1
	63	NA	24.4
	68	NA	24.6
2	5'000#	31.20	23.6
	4'600#	30.55	24.2
	4'000#	31.74	23.8
	496	NA	24.1
	460	NA	23.7
	408	NA	23.4
	51	NA	23.9
	44	NA	24.3
	40	NA	23.9
3	4200#	31	23.8
	3300#	32.19	24.2
	4900#	30.95	24.1
	423	NA	24.4
	336	NA	24.6
	490	NA	24.2
	33	NA	24.2
	29	NA	23.9
	47	NA	24.5

Table 2. Results of customised *M. chimaera* RT-PCR performed in 3 independent experiments, in triplicate. The red box highlights the replicate used to calculate the LoD.
#Deduced from the previous serial dilution.
NA = not amplificated.

other commercial RT-PCR for Mycobacterium Tuberculosis detection[20], reflecting a low sensitivity of this system to identify *M. chimaera*. Furthermore, a limitation of this approach to calculating the LoD is the difficulty of standardizing inoculum for mycobacteria, reflected in low inter-experiment reproducibility.

The specificity of the assay was assessed on 161 water samples that were culture-negative for *M. chimaera*, including 41 water samples positive for other NTM (1 *M. chelonae*, 19 *M. gordonae* and 21 *M. paragordonae*). Specificity was 98.8%, indicating that a positive result nearly always confirms the presence of viable *M. chimaera*. Only two samples with PCR positive but culture-negative results were detected; however, these samples had been taken from an HCU characterised by intermittent culture positivity (over the 3-year monitoring period).

In conclusion, this study showed that the detection of *M. chimaera* with RT-PCR from concentrated and decontaminated HCU-water is be able to support culture-based methods, reducing the time taken to shut down HCUs in the event of positivity. In addition, the water samples used in this study were concentrated 1:100 as per protocol leading to detection of *M.chimaera* DNA starting at 29 CFU/ml from water circulating in medical devices.

Data availability

All data analysed are reported in the result section and the dataset used during the current study is available from the corresponding author on reasonable request.

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Author contributions

F.B. and P.D.M. conceived and designed the experiments. M.B., M.F., V.F., C.M.C.P. and A.P. performed the experiments and curated the data. F.B., M.B., F.S. and M.F. analyzed the data. F.B. and P.D.M. wrote and revised the paper. P.D.M. supervised the project.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

No ethical statements are required as this study was conducted on environmental specimens.

Additional information

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