A target-specific assay for rapid and quantitative detection of *Mycobacterium chimaera* DNA in environmental and clinical specimens

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Running Head: Molecular detection of *M. chimaera*

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Abstract. *Mycobacterium chimaera* is an opportunistic environmental mycobacterium, belonging to the *Mycobacterium intracellulare* complex. Although most commonly associated with pulmonary disease, there has been growing awareness of invasive *M. chimaera* infections following cardiac surgery. Investigations suggest world-wide spread of a specific *M. chimaera* clone, associated with contaminated hospital heater-cooler units used during the surgery. Given the global dissemination of this clone, its potential to cause invasive disease, and the laboriousness of current culture-based diagnostic methods, there is a pressing need to develop rapid and accurate diagnostic assays, specific for *M. chimaera*. Here, we assessed 354 mycobacterial genome sequences and confirmed that *M. chimaera* is a phylogenetically coherent group. *In silico* comparisons indicated six DNA regions present only in *M. chimaera*. We targeted one of these regions and developed a TaqMan qPCR assay for *M. chimaera* with a detection limit of 10 CFU in whole blood. *In vitro* screening against DNA extracted from 40 other mycobacteria and 22 bacterial species from 21 diverse genera confirmed *in silico* predicted specificity for *M. chimaera*. Screening 33 water samples from heater cooler units with this assay highlighted the increased sensitivity of PCR compared to culture, with 15 of 23 culture negative samples positive by *M. chimaera* qPCR. We have thus developed a robust molecular assay that can be readily and rapidly deployed to screen clinical and environmental specimens for *M. chimaera*.

Introduction.

Mycobacterium chimaera is an environmental mycobacterium and infrequent pathogen, most commonly linked with pulmonary disease (1-8). Interest in *M. chimaera* has heightened with global reports of invasive infections (including endocarditis and vascular graft infections associated with the use of LivaNova PLC (formerly Sorin Group Deutschland GmbH) Stöckert 3T heater-cooler units during cardiac surgery. The most plausible hypothesis for this widespread contamination is a point-source outbreak, although the underlying causative factors are not currently known (9-16). Phylogenetic comparisons of 16S–23S rRNA internal transcribed spacer (ITS) sequences, and/or partial *rpoB* or *hsp65* sequences (2, 5-7, 17, 18) suggest *M. chimaera* as a distinct entity within the *M. intracelluare* complex (6) and two recent population genomic analyses have confirmed this relationship (8, 13). The complete 6,593,403 bp genome sequence of *M. chimaera* ANZ045 revealed a single circular 6,078,672 bp chromosome and five circular plasmids ranging in size from 21,123 bp to 324,321 (8). *M. chimaera* is slow-growing, therefore current culture-based laboratory methods, followed by Sanger sequencing of amplicons for one or more combinations of conserved sequence regions, or line-probe hybridization assays are not amenable to timely and specific detection of this pathogen. This delay carries significant clinical, health provision, and medico-legal implications as patients may be exposed to contaminated machines during this turn-around time of up to 6-8 weeks. A rapid and reliable diagnostic tool is urgently needed to support clinical management of patients and to establish the efficacy of heater-cooler unit decontamination procedures. Here we addressed this issue by using comparative genomics to identify DNA sequences present in *M. chimaera* and absent from other mycobacteria. We describe the initial development and validation of a sensitive, specific and quantitative PCR assay for identification of *M. chimaera* in both clinical and environmental samples.

65 **Materials and methods**.

66 ***Bacterial strains and genome sequences.*** *Mycobacterium chimaera* strain DMG1600125 (a 2016
67 HCU isolate from New Zealand) was used for spiking experiments (8). The mycobacterial genome
68 sequences used in this study are listed in Table S1. *M. chimaera* was grown on Brown and Buckle
69 whole-egg media, Middlebrook 7H9 broth or Middlebrook 7H10 agar (Becton Dickinson)
70 supplemented with 10% (v/v) oleic acid albumin dextrose complex (OADC; Difco) or Middlebrook
71 7H10 agar. Cultures were incubated without shaking at 37°C. *M. chimaera* colony counts were
72 obtained by spotting 3 µL microliter volumes of six, 10-fold serial dilutions of a *M. chimaera*
73 culture suspensions in quintuplicate on two Middlebrook 7H10 agar plates. The colonies were
74 counted after incubation for four weeks at 37°C.
75
76 ***Genomic DNA extraction methods, M. chimaera culture and environmental isolation.*** Purified
77 *M. chimaera* genomic DNA for TaqMan assay validation was extracted from 50 mg wet weight cell
78 pellets as described (19) and measured by fluorimetry using the Qubit and the *High Sensitivity*
79 DNA kit (Thermofisher). For spiking experiments in blood, *M. chimaera* DNA was extracted from
80 100 µL volumes of whole blood, using the Qiagen Blood & Tissue DNA extraction kit. Purified
81 DNA was eluted from the columns in a 200 µL volume of 10 mM Tris (pH 8.0) (Qiagen). Total
82 bacteria were concentrated from 30-1000 mL volumes of water collected from heater-cooler units
83 by filtration through 47 mm, 0.22 µM mixed cellulose ester Millipore membranes. Immediately
84 after filtration, membranes were aseptically placed in sterile 50 ml plastic tubes and stored at -70°C.
85 DNA was extracted from membrane concentrate using the MoBio PowerWater DNA isolation kit
86 following the manufacturer's instructions (MoBio) with an additional physical disruption step
87 consisting of 2 x 20 sec at 5000 rpm in a Precellys 24 tissue homogenizer. To prevent cross
88 contamination, a sterilized filtration device was used for each sample and sterile, distilled water
89 extraction blanks were filtered and processed (100 mL volumes) at a frequency of one for every 10

test samples. Culture isolation of *M. chimaera* from 50 mL volumes of water samples was undertaken as described (20).

Population structure and phylogenetic analysis. Snippy v3.1 (https://github.com/tseemann/snippy) was used to align Illumina sequence read data or *de novo* assembled contigs from *M. chimaera* and related mycobacterial genomes against the fully-assembled, complete MC_ANZ045 reference genome to call core genome single nucleotide polymorphism (SNP) differences and generate pairwise sequence alignments. Hierarchical Bayesian clustering (hierBAPS) was performed using these core whole-genome SNP alignments as input to assess population structure (a prior of 6 depth levels and a maximum of 20 clusters was specified) (21), with phylogenies inferred using FastTree v2.1.8 under a GTR model of nucleotide substitution (22). Pairwise SNP analysis between groups of genomes was performed using a custom R script (https://github.com/MDU-PHL/pairwise_snp_differences). Recombination detection was performed using ClonalFrameML v1.7 (23).

In silico subtractive hybridization and target identification. To identify regions of DNA present in *M. chimaera* but absent from other mycobacteria, the Illumina sequence reads of 46 *M. chimaera* isolates from Australia and New Zealand and eight publicly available *M. intracellulare* genomes (Table S1) were aligned using BWA MEM v0.7.15-r1140 (https://arxiv.org/abs/1303.3997) to a complete *M. chimaera* reference genome (MC_ANZ045) (8). The read depth at each position was examined to identify those positions in the reference genome that were present across all *M. chimaera* isolates but absent from all *M. intracellulare* genomes. These genomic regions were extracted from MC_ANZ045 and compared against the NCBI Genbank non-redundant (*nt*) nucleotide database using NCBI BLAST v2.5.0 (CITE?) with parameters *-remote -max_target_seqs 100 -task blastn -outfmt "6 std qcovs staxid ssciname"*. Resulting BLAST hits that were missing a

taxon name were retrieved from the NCBI taxonomy database using the taxon id. Ignoring BLAST hits against bonafide *M. chimaera* sequences, the query alignment positions for every hit were extracted and were used to obtain all the sequence segments that had no hits against the Genbank *nt* database and that were greater than 500 bp in length. For this, *bedtools complement* and *getfasta* tools were used (24). The sequence segments thus obtained were considered candidate *M. chimaera*-specific genomic regions. The presence of these regions across a wider collection of *M. chimaera* was assessed by downloading all *M. chimaera* genome sequence reads present in the NCBI sequence read archive SRA as of October 2016 (Table S1) and processing through the Nullarbor pipeline v1.2 (https://github.com/tseemann/nullarbor). The output information was used to filter out poor quality or non-*M. chimaera* reads sets based on G+C content significantly below 66%, an average read depth below 30, a total contig length above 8Mb, predicted rRNA genes greater than four, or a total of sequence aligned to the reference genome below 70% (Table S1). Using Snippy again, all *M. chimaera* genomes identified above were mapped to a version of the MC_ANZ045 reference genome in which the non-*M. chimaera*-specific sequence regions had been hard masked. The resulting multiple sequence alignment was parsed using a custom Perl script to identify those *M. chimaera*-specific regions that were present in all *M. chimaera* genomes. These DNA sequences were inspected further for development of *M. chimaera* TaqMan PCR diagnostic assays. TaqMan primers and probes (Sigma Oligonucleotides) were designed using Primer3 (25) and Primer-BLAST against NCBI *nt* database was used to check that the primers and probes designed were specific to *M. chimaera*. TaqMan probe 1970-P were labeled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and a nonfluorescent quencher at the 3′ end (Sigma Oligonucleotides). To assess the context of these *M. chimaera*-specific regions, AlienHunter v1.4 was used to screen the MC_ANZ045 genome for DNA compositional bias, indicative of horizontally acquired DNA (26).

TaqMan quantitative PCR. TaqMan PCR mixtures contained 2 µl of template DNA, 0.4-µM concentrations of each primer, a 0.2 µM concentration of the probe, SensiFAST Probe Lo-ROX (1x) mix (Bioline), and TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems) in a total volume of 20 µl. Amplification and detection were performed with the Mx3005P (Stratagene) using the following program: 40 cycles of 95°C for 10 s and 60°C for 20 s. DNA extracts were tested in at least duplicate, and negative and positive template controls were included in each run. Standard curves were prepared using eight, 10-fold serial dilutions of *M. chimaera* genomic DNA at an initial concentration of 120 ng/µL, tested in triplicate. The percentage PCR amplification efficiency (E) for the TaqMan assay was calculated from the slope (C) of the standard curve E = (10^(-1/C))*100. Cycle threshold values for unknown samples were converted to genome equivalents by interpolation, with reference to the standard curve of Ct versus dilutions of known concentrations of *M. chimaera* genomic DNA. The mass in femtograms of a single *M. chimaera* genome was estimated as 6.59 fg, using the formula M = (N)*(1.096e-21), where M = mass of the single double-stranded *M. chimaera* NZ045 reference genome and N = 6593403, which is the length of the *M. chimaera* NZ045 reference genome, and assuming the average MW of a double-stranded DNA molecule is 660 g/mol. Analyses were performed using Graphpad Prism v6.0h.

Results

Assessment of* M. chimaera *population structure. To identify DNA segments present only in *M. chimaera* genomes we first assessed the phylogenetic coherence of "*M. chimaera*" as a species. Using 96 mycobacterial genome sequences, comprising 63 *M. chimaera* genomes from North America, Australia, and New Zealand, and 33 other related, publicly available mycobacteria from the *Mycobacterium avium-intracelluare* complex, we conducted whole genome pairwise comparisons of the 96 taxa to the *M. chimaera* ANZ045 complete reference chromosome. The 63

M. chimaera genomes included 49 HCU-associated and 14 previously described patient isolates, not all of which were associated with Stöckert 3T HCU contamination (8, 12). These comparisons identified 448,878 variable nucleotide positions in a 2,340,885 bp core genome. A robust phylogeny inferred from the alignments strongly suggested that *M. chimaera* forms a monophyletic lineage within the *M. intracellulare* complex (Fig. 1A) (8, 13). Bayesian analysis of population structure (BAPS) using these same data confirmed this clustering (Fig. 1A). Interestingly, this assessment indicated that a publicly available isolate originally identified as *Mycobacterium intracellulare* (strain MIN_052511_1280) was in fact *M. chimaera*. The mean number of SNPs between any pair of the 63 *M. chimaera* isolates, and MIN_052511_1280 (BAPS-3), not adjusted for recombination, was 115 SNPs (range: 1 - 3,024 and IQR: 13 - 31), highlighting restricted core genome variation within this species, particularly given the large 6.5 Mb genome size. In comparison, the mean number of SNPs between 15 *M. intracellulare*-complex genomes (BAPS-2) was 24,134 SNPs (range: 13 - 39,109 and IQR: 14,780 - 33,138) (Fig. 1A). We then extended this analysis to assess an additional 257 publicly available *M. chimaera* and related mycobacterial genome sequences (Table S1). Pairwise whole genome comparisons of this larger data set were performed against the ANZ045 reference genome. Population structure analysis indicated 303 mycobacterial genomes fell within BAPS-3 (Table S1). Pairwise comparisons were again performed against the ANZ045 using only these 303 genomes, with five other genome sequences from BAPS-2 included for context. The alignment was filtered to remove sites from the alignment affected by recombination and a phylogeny was inferred from the resulting 10,166 variable nucleotide positions (Fig. S1. Fig. 2). The mean number of SNPs between the 303 genomes was 268 (range: 0 - 3,211 and IQR: 9 - 62). This analysis confirmed that *M. chimaera* does indeed form a monophyletic lineage, providing a robust genetic definition for the species. As previously reported, HCU-associated isolates from around the world formed a distinct sub-clade within this lineage (Fig. 2) (8, 13).

In silico *genome comparisons to identify* M. chimaera *specific sequences.* A subset of 46 genome *M. chimaera* genome sequences as defined above became the 'training set' to find DNA segments present only in *M. chimaera* (Fig. 2, Table S1). Mapping of DNA sequence reads to the ANZ045 reference genome (refer methods) allowed the identification of 159 genomic segments >500 bp in length and covering 510,924 bp that were present across the 46 *M. chimaera* isolates and absent from eight *M. intracellulare* isolates (Fig. 1A). BLAST comparisons of the 159 segments against all entries in the NCBI Genbank *nt* database and removal of any non-*M. chimaera* specific sequence reduced the number to 37 segments (covering a total of 37,890 bps). A larger validation set comprising the 63 *M. chimaera* genomes described in Fig. 1A and 242 additional, publicly available *M. chimaera* genomes that satisfied our above phylogenomic inclusion criteria was then screened (Table S1). Six of the 37 *M. chimaera*-specific regions (SR), covering a total of 8,292 bp, were present in all 305 *M. chimaera* genomes (Fig. 1B). The six SRs ranged in length from 531 bp to 4,641 bp, the majority overlapping predicted chromosomal protein-coding sequences (Fig. 1B, Table 1). Inferred functions of these CDS are summarized (Table 1). The regions were scanned for sequence polymorphisms and one of these regions (SR1) that was 100% conserved among all *M. chimaera*, was selected as a template for the design of a TaqMan assay (Fig. 2, Table 2). SR1 spans two predicted CDS that DNA composition analysis and gene annotation predicted lay within a 35 - kb putative prophage or integrative mobile element. A 79 bp TaqMan amplicon (assay ID: 1970) was designed within a 2934 bp CDS (predicted function: unknown) (Table 2).

TaqMan assay specificity testing. The above *in silico* analyses predicted the TaqMan assay would be diagnostic for the presence of *M. chimaera*. To test this prediction, DNA was prepared from 42 mycobacteria (including two *M. chimaera* isolates) and 22 other bacteria from 21 different genera. A pan-bacterial 16S rRNA PCR was first performed to ensure detectable bacterial DNA was

present. All 64 DNA samples were positive by 16S rRNA PCR (data not shown) but only the two *M. chimaera* isolates were 1970-P TaqMan assay positive, supporting the *in silico* predictions that these assays are specific for *M. chimaera* (Table S2).

TaqMan assay efficiency and sensitivity testing. To establish the limit of detection and amplification efficiency for the 1970-P TaqMan assay, 10-fold serial dilutions of purified *M. chimaera* ANZ045 genomic DNA were tested in triplicate. The 1970-P assay showed excellent performance characteristics, with a very good linear response across five orders of magnitude, R^2 values >0.99, amplification efficiencies of 94%, and a detection limit around 20 genome equivalents (Fig. 3A). The detection limit was also assessed using dilutions of *M. chimaera* culture spiked into whole blood. Again, the assay showed excellent performance characteristics in this simulated clinical condition, with an absolute detection limit around 10 CFU (equivalent to 100 CFU/mL of blood). These experiments indicate 1970-P is a suitable qPCR assay for sensitive and quantitative detection of *M. chimaera* DNA.

Detection of M. chimaera DNA in environmental samples. A key requirement for this assay is the ability to screen water and biofilm samples from heater-cooler units for *M. chimaera*, to determine if maintenance procedures have removed the bacteria from contaminated units, or prevented contamination. Having assessed the sensitivity and specificity of the assay with laboratory-prepared samples, we next explored performance with environmental samples. We screened concentrates from 33 water samples obtained from heater-cooler units at seven hospitals and HCU distributors in our region, that had been assessed by culture for *M. chimaera*. A total of 25 of 33 samples were positive by 1970-P TaqMan PCR, with estimated *M. chimaera* concentrations ranging from 2-102,000 GE/mL of water (Table 3). Using the culture results as a 'gold standard', the negative predictive value for the TaqMan assays was high (100%) with all eight culture positive samples

also positive 1970-P TaqMan PCR (Table 4). However, there was poor correspondence between culture negative samples and qPCR. Fifteen samples negative by culture returned 1970-P TaqMan positive results, with Ct values for some of these samples less than 24, indicating high *M. chimaera* concentrations above 10,000 GE per milliliter of original sample (Table 3). Heterotrophic colony count (HCC) at 37ºC is used as a general indicator of water cleanliness, and in some settings may be used as a surrogate indicator of decontamination effectiveness (27). However, we observed a poor correlation between HCC and the presence of *M. chimaera* as measured by qPCR (Spearman's ρ=0.2813, p=0.1128) (Table 3), suggesting that HCC is not a suitable surrogate for the presence or absence of *M. chimaera*.

Discussion

Since 2012 there have been a small but increasing number of case reports of invasive infection with *M. chimaera* in individuals who have undergone surgical procedures requiring cardiac bypass (8, 10-16). Almost all cases have involved placement of prosthetic valves or other prosthetic material and are linked to use of a specific type of heater cooler unit (HCU) in the bypass procedure (8, 13, 14). As contamination of these HCUs may have occurred at or near the time of manufacture and the machines are widely exported, exposure to *M. chimaera* during cardiac bypass surgery is an emerging issue in infection control that is not restricted by region or country (8). However, while it appears that generation of aerosols when machines are contaminated may be relatively common, so far the likelihood of infection for any exposed individual is very low (13). From a clinical perspective, this poses a major diagnostic challenge. Post-cardiac surgery *M. chimaera* infection has a long incubation period, non-specific symptoms and can be misdiagnosed as a steroid-requiring inflammatory condition with potentially disastrous consequences (10, 15) . Moreover, there is a significant case fatality rate even when correctly identified and current opinion is that early accurate diagnosis will be key to achieving the best treatment outcomes (28). Given the non-

specific symptoms and low prior probability of infection in a large exposed population, clinicians urgently need access to a specific and sensitive test for *M. chimaera* cardiac and extra-cardiac infection. Infection control practitioners have a different but equally challenging problem with respect to surveying and cleaning contaminated HCUs. In this report, we describe and validate a DNA target that is diagnostic for the presence of *M. chimaera*. We have shown under simulated conditions that it can accurately detect *M. chimaera* in human blood samples at very low concentrations and outperform culture in detecting *M. chimaera* in specimens obtained from contaminated HCUs.

The European Centre for Disease Prevention and Control recommends *M. chimaera* identification is performed by sequencing at least two conserved fragments among 16S–23S rRNA ITS, 16SrRNA, *rpoB* and *hsp65* [21,22]. Some laboratories have also used the MIN-2 probe in the INNO-LiPA Mycobacteria v2 line probe assay (6, 18). Here we simplify this suite of tests, with a *M. chimaera*-specific PCR assay, that has the advantages of providing a rapid yes/no result and an estimate of bacterial concentration. The test could be further enhanced with multiple DNA targets. The other five *M. chimaera*-specific regions reported here could be used to develop additional diagnostic targets (Fig. 1B). We envisage that this test will be used in conjunction with efforts to culture *M. chimaera* from specimens, as isolates are required for WGS to establish the genetic relatedness of isolates, and potentially for antimicrobial susceptibility testing (although antimicrobial resistance is not thought to be a major problem). As a previous risk assessment by Public Health England suggested a possible legionellosis risk for staff and patients, we propose that our assay should form part of a 'HCU panel' along with *Legionella* PCR (28, 29). While we have designed an assay to detect all *M. chimaera*, it may also be possible to detect specific *M. chimaera* lineages. For instance, it may be possible to use DNA deletion polymorphisms to discriminate

among intra-species lineages, as in the *Mycobacterium tuberculosis* complex (30). We are currently exploring this possibility.

The infection risk posed by the water reservoirs within HCUs and the need for regular maintenance has been long recognized (29). Heterotrophic colony counts (HCC) are being considered as surrogates to assess the microbiological quality of HCUs (27, 31). We (like others) found a poor correlation between the presence of *M. chimaera* and HCC in water samples from HCUs (27), with examples of *M. chimaera* concentrations of 60,000 GE/mL when HCC were below the limit of detection (Table 3). More evaluation of the 1970-P assay is required, but our data suggest HCC may have an unacceptably high false-negative rate, which significantly reduces its utility for measuring the effectiveness of HCU decontamination procedures.

Screening HCU water samples with our TaqMan assay indicated the widespread presence of *M. chimaera* and a poor correlation with culture. There are several potential explanations for these observations. Despite the extensive *in silico* assessments, it is possible that the qPCR assay lacks specificity for *M. chimaera*, or that the culture method lacks sensitivity, or perhaps DNA from *M. chimaera* is still present but source organisms are no longer viable. Given the extensive *in silico* validation undertaken here to ensure target specificity, and the high prior probability that these HCU water samples contained *M. chimaera*, the discrepancy between culture and qPCR might best explained by either lower sensitivity of the mycobacterial culture method or PCR detection of intact DNA from non-viable *M. chimaera*. The later explanation is perhaps the most likely given that some of these PCR positive-culture negative samples were obtained from HCUs subjected to extensive decontamination procedures involving extended heating above 70ºC.

In summary, we have developed a new diagnostic tool for rapid, sensitive and specific detection of *M. chimaera* to help address the urgent need to screen patient and HCU samples.

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References

1. **Alhanna J, Purucker M, Steppert C, Grigull-Daborn A, Schiffel G, Gruber H, Borgmann S.** 2012. *Mycobacterium chimaera* causes tuberculosis-like infection in a male patient with anorexia nervosa. Int J Eat Disord **45:**450-452.
2. **Bills ND, Hinrichs SH, Aden TA, Wickert RS, Iwen PC.** 2009. Molecular identification of *Mycobacterium chimaera* as a cause of infection in a patient with chronic obstructive pulmonary disease. Diagn Microbiol Infect Dis **63:**292-295.
3. **Cohen-Bacrie S, David M, Stremler N, Dubus JC, Rolain JM, Drancourt M.** 2011. *Mycobacterium chimaera* pulmonary infection complicating cystic fibrosis: a case report. J Med Case Rep **5:**473.
4. **Mac Aogain M, Roycroft E, Raftery P, Mok S, Fitzgibbon M, Rogers TR.** 2015. Draft Genome Sequences of Three *Mycobacterium chimaera* Respiratory Isolates. Genome Announc **3**.
5. **Moon SM, Kim SY, Jhun BW, Lee H, Park HY, Jeon K, Huh HJ, Ki CS, Lee NY, Shin SJ, Koh WJ.** 2016. Clinical characteristics and treatment outcomes of pulmonary disease caused by *Mycobacterium chimaera*. Diagn Microbiol Infect Dis **86:**382-384.

6. **Tortoli E, Rindi L, Garcia MJ, Chiaradonna P, Dei R, Garzelli C, Kroppenstedt RM, Lari N, Mattei R, Mariottini A, Mazzarelli G, Murcia MI, Nanetti A, Piccoli P, Scarparo C.** 2004. Proposal to elevate the genetic variant MAC-A, included in the Mycobacterium avium complex, to species rank as *Mycobacterium chimaera* sp. nov. Int J Syst Evol Microbiol **54:**1277-1285.

7. **Schweickert B, Goldenberg O, Richter E, Gobel UB, Petrich A, Buchholz P, Moter A.** 2008. Occurrence and clinical relevance of *Mycobacterium chimaera* sp. nov., Germany. Emerg Infect Dis **14:**1443-1446.

8. **Williamson D, Howden B, Stinear T.** 2017. Spread of *Mycobacterium chimaera* from Units Used in Cardiac Surgery. New Eng J Med **In press**.

9. **Zweifel SA, Mihic-Probst D, Curcio CA, Barthelmes D, Thielken A, Keller PM, Hasse B, Boni C.** 2016. Clinical and Histopathologic Ocular Findings in Disseminated *Mycobacterium chimaera* Infection after Cardiothoracic Surgery. Ophthalmology doi:10.1016/j.ophtha.2016.09.032.

10. **Tan N, Sampath R, Abu Saleh OM, Tweet MS, Jevremovic D, Alniemi S, Wengenack NL, Sampathkumar P, Badley AD.** 2016. Disseminated *Mycobacterium chimaera* Infection After Cardiothoracic Surgery. Open Forum Infect Dis **3:**ofw131.

11. **Sommerstein R, Ruegg C, Kohler P, Bloemberg G, Kuster SP, Sax H.** 2016. Transmission of *Mycobacterium chimaera* from Heater-Cooler Units during Cardiac Surgery despite an Ultraclean Air Ventilation System. Emerg Infect Dis **22:**1008-1013.

12. **Perkins KM, Lawsin A, Hasan NA, Strong M, Halpin AL, Rodger RR, Moulton-Meissner H, Crist MB, Schwartz S, Marders J, Daley CL, Salfinger M, Perz JF.** 2016. Notes from the Field: *Mycobacterium chimaera* Contamination of Heater-Cooler Devices Used in Cardiac Surgery - United States. MMWR Morb Mortal Wkly Rep **65:**1117-1118.

13. **Chand M, Lamagni T, Kranzer K, Hedge J, Moore G, Parks S, Collins S, Del Ojo Elias C, Ahmed N, Brown T, Smith EG, Hoffman P, Kirwan P, Mason B, Smith-Palmer A, Veal P, Lalor MK, Bennett A, Walker J, Yeap A, Isidro Carrion Martin A, Dolan G, Bhatt S, Skingsley A, Charlett A, Pearce D, Russell K, Kendall S, Klein AA, Robins S, Schelenz S, Newsholme W, Thomas S, Collyns T, Davies E, McMenamin J, Doherty L, Peto TE, Crook D, Zambon M, Phin N.** 2016. Insidious risk of severe *Mycobacterium chimaera* infection in cardiac surgery patients. Clin Infect Dis doi:10.1093/cid/ciw754.

14. **Sax H, Bloemberg G, Hasse B, Sommerstein R, Kohler P, Achermann Y, Rossle M, Falk V, Kuster SP, Bottger EC, Weber R.** 2015. Prolonged Outbreak of *Mycobacterium chimaera* Infection After Open-Chest Heart Surgery. Clin Infect Dis **61:**67-75.

15. **Kohler P, Kuster SP, Bloemberg G, Schulthess B, Frank M, Tanner FC, Rossle M, Boni C, Falk V, Wilhelm MJ, Sommerstein R, Achermann Y, Ten Oever J, Debast SB, Wolfhagen MJ, Brandon Bravo Bruinsma GJ, Vos MC, Bogers A, Serr A, Beyersdorf F, Sax H, Bottger EC, Weber R, van Ingen J, Wagner D, Hasse B.** 2015. Healthcare-associated prosthetic heart valve, aortic vascular graft, and disseminated *Mycobacterium chimaera* infections subsequent to open heart surgery. Eur Heart J **36:**2745-2753.

16. **Achermann Y, Rossle M, Hoffmann M, Deggim V, Kuster S, Zimmermann DR, Bloemberg G, Hombach M, Hasse B.** 2013. Prosthetic valve endocarditis and bloodstream infection due to *Mycobacterium chimaera*. J Clin Microbiol **51:**1769-1773.

17. **van Ingen J, Hoefsloot W, Buijtels PC, Tortoli E, Supply P, Dekhuijzen PN, Boeree MJ, van Soolingen D.** 2012. Characterization of a novel variant of *Mycobacterium chimaera*. J Med Microbiol **61:**1234-1239.

18. **Lebrun L, Weill FX, Lafendi L, Houriez F, Casanova F, Gutierrez MC, Ingrand D, Lagrange P, Vincent V, Herrmann JL.** 2005. Use of the INNO-LiPA-MYCOBACTERIA assay (version 2) for identification of Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum complex isolates. J Clin Microbiol **43:**2567-2574.

19. **Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, Rhodes M, Kator H, Colorni A, Jenkin GA, Stinear T.** 2007. Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common Mycobacterium marinum progenitor. J Bacteriol **189:**2021-2029.

20. **PHE.** 2016. Protocol for Environmental Sampling, Processing and Culturing of Water and Air Samples for the Isolation of Slow-Growing Mycobacteria. Health Do, Public Health England, London, UK. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/540325/Air_water_environmental_sampling_SOP_V2.pdf.

21. **Cheng L, Connor TR, Sirén J, Aanensen DM, Corander J.** 2013. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. Molecular biology and evolution **30:**1224-1228.

22. **Price MN, Dehal PS, Arkin AP.** 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol **26:**1641-1650.

23. **Didelot X, Wilson DJ.** 2015. ClonalFrameML: efficient inference of recombination in whole bacterial genomes. PLoS Comput Biol **11:**e1004041.

24. **Quinlan AR, Hall IM.** 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics **26:**841-842.

25. **Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG.** 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res **40:**e115.

26. **Vernikos GS, Parkhill J.** 2006. Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. Bioinformatics **22:**2196-2203.

27. **PHLN.** 2017. Public Health Laboratory Network: guidance regarding *Mycobacterium chimaera* and heater-cooler units. Department of Health, Australian Government, Canberra, Australia. http://www.health.gov.au/internet/main/publishing.nsf/Content/83DA1AD46068E894CA258082001C86F0/$File/PHLN-guidance-Mycobacterium-chimaera.pdf.

28. **PHE.** 2015. Infections associated with heater cooler units used in cardiopulmonary bypass and ECMO: information for healthcare providers in England. Public Health England, London, UK. https://www.gov.uk/government/publications/infections-associated-with-heater-cooler-units-used-in-cardiopulmonary-bypass-and-ecmo.

29. **Weitkemper HH, Spilker A, Knobl HJ, Korfer R.** 2002. The heater-cooler unit--a conceivable source of infection. J Extra Corpor Technol **34:**276-280.

30. **Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM, Pym AS, Samper S, van Soolingen D, Cole ST.** 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci U S A **99:**3684-3689.

31. **Anon.** 2016. Nontuberculous Mycobacterium (NTM) Infections Associated with Heater-Cooler Devices (HCD) during Cardiothoracic Surgery. Administration USFaD, FDA, USA. http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/MedicalDevices/MedicalDevicesAdvisoryCommittee/CirculatorySystemDevicesPanel/UCM503716.pdf.

32. **Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA.** 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics **12:**402.

Figures:

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Supplementary material

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Fig. 1

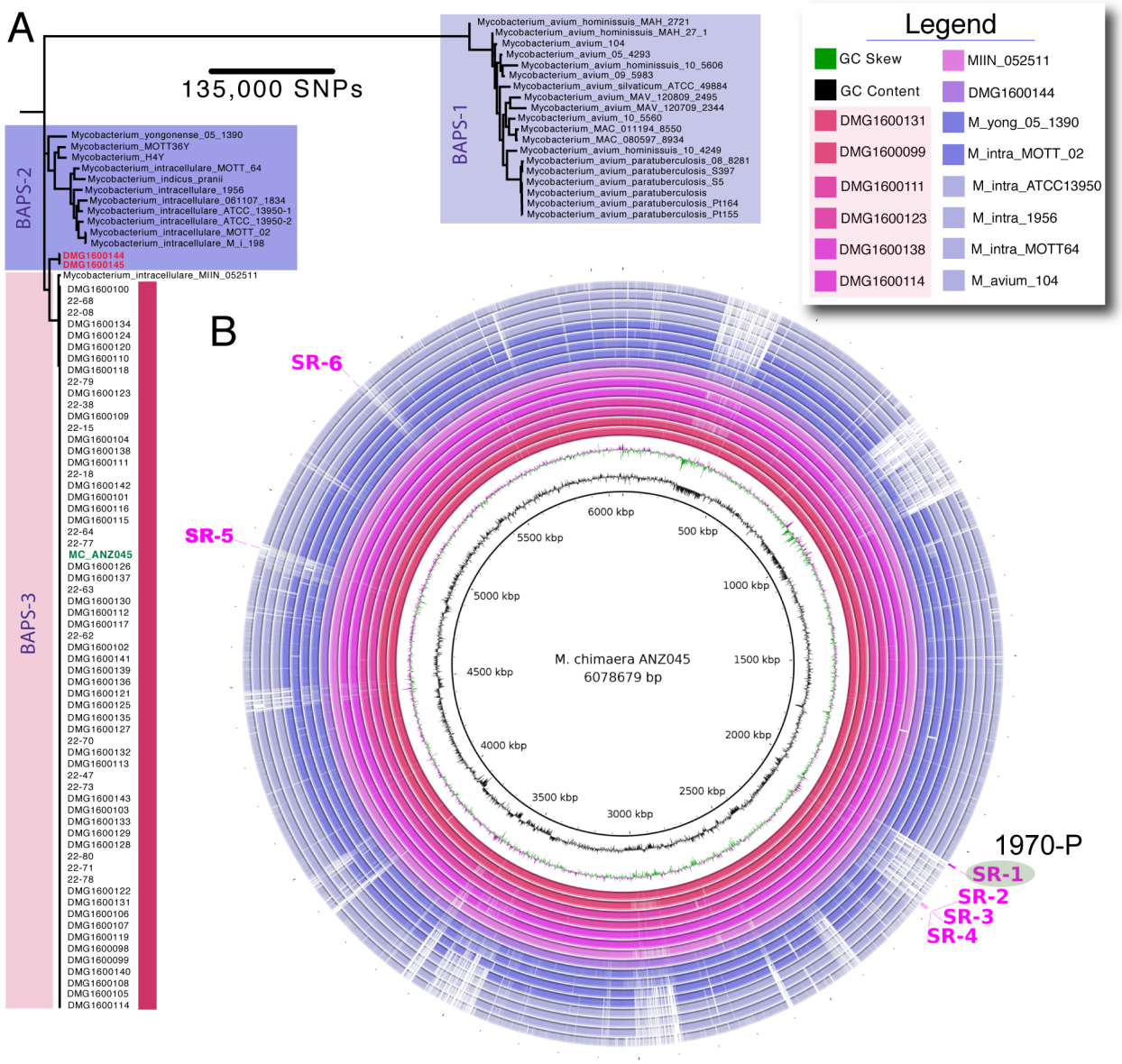


Fig. 1. Phylogenetic and population structure analysis of *M. chimaera* (A): Core genome maximum likelihood phylogeny of 63 *M. chimaera* and 33 other, related mycobacteria, based on alignment of 448,878 variable nucleotide positions. The tree was inferred with FastTree using a GTR model of nucleotide substitution. All major branches had FastTree support values >0.9. Lineages are coloured and BAPS group designations are indicated. The scale bar indicates the number of SNPs represented by the horizontal branches. The location of ANZ045 *M. chimaera* reference genome is shown in green typeface. (B) Visualization using the Blast Ring Image Generator (32) of DNA:DNA whole genome comparisons among a subset of mycobacterial genomes used to identify *M. chimaera* specific regions. ANZ045 *M. chimaera* reference chromosome is depicted by the inner black circle. The identity of the subsequent rings is given in the legend. Annotations on the outer most ring show the location of the seven *M. chimaera*-specific regions, highlighting the region targeted for TaqMan PCR assay development.

Fig. 2

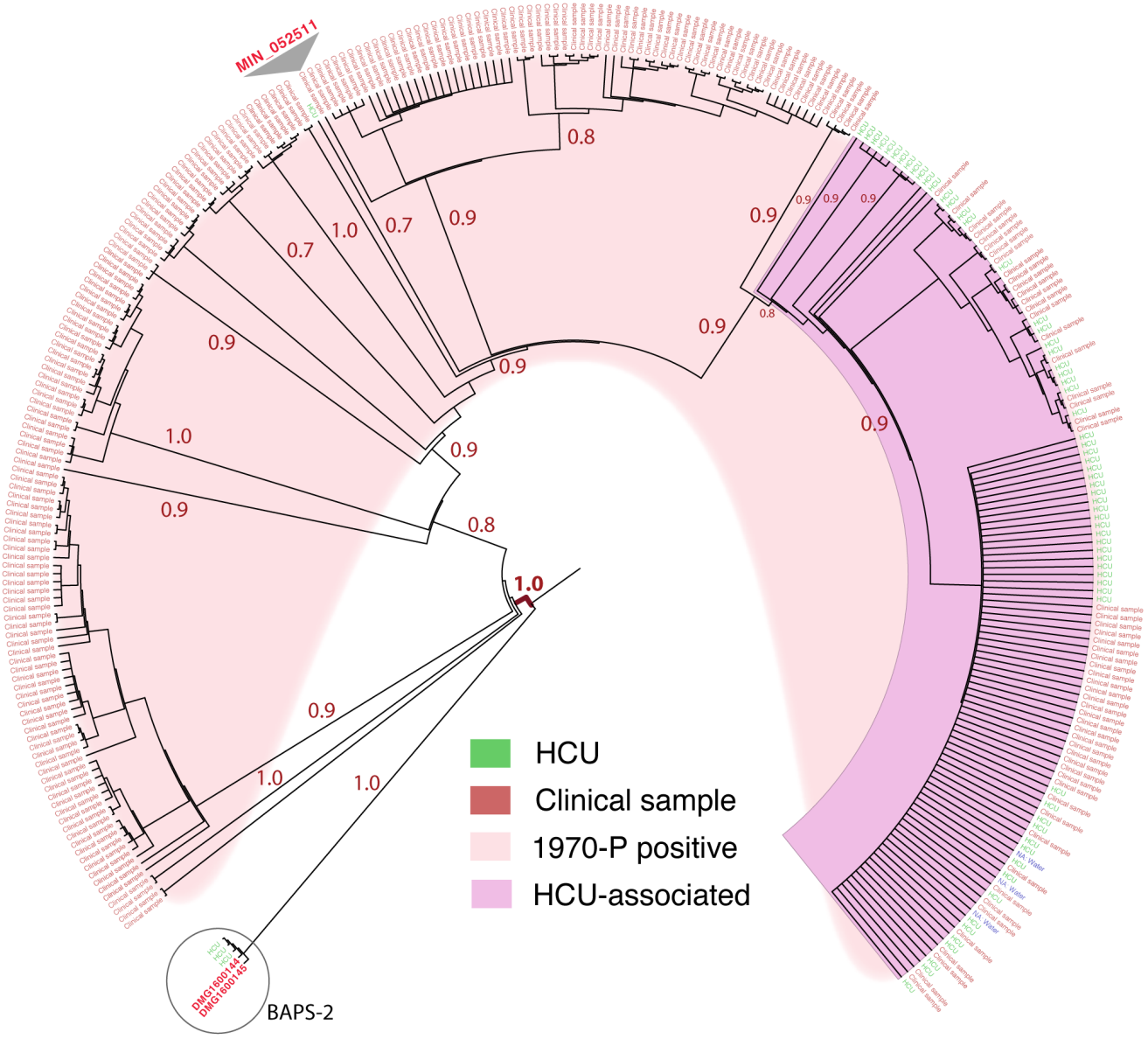
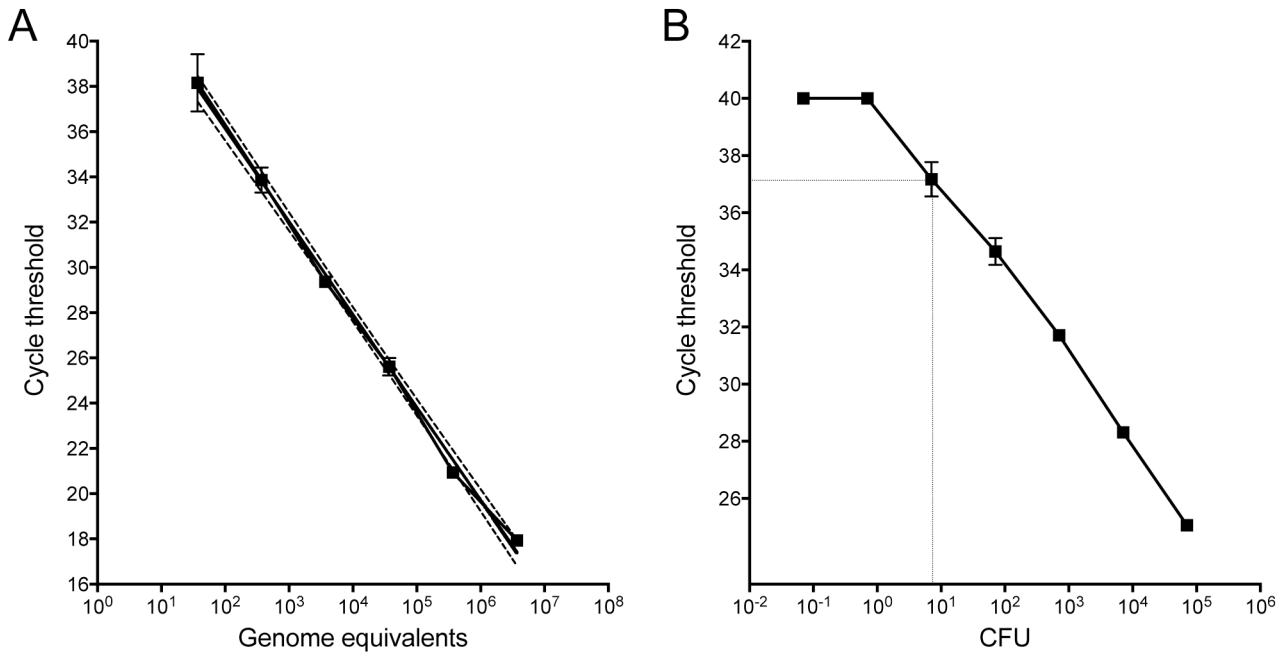


Fig. 2. Focused phylogenetic analysis of 303 *M. chimaera* genomes. (A): Core genome maximum likelihood phylogeny of 303 *M. chimaera* (BAPS-3) and five other, related mycobacteria (BAPS-2), based on alignment of 10,166 variable nucleotide positions (recombination removed). The tree was inferred with FastTree using a GTR model of nucleotide substitution and rooted using DMG1600144 (BAPS-2, encircled) as an outgroup. The FastTree support values for major branches are indicated. Branch lengths have been transformed and they are proportional but not to scale. The location within the phylogeny of a sequence identified previously as *M. intracellulare* (MIN_052511) is indicated.

484 **Fig. 3**



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486 **Fig. 3. Sensitivity testing of 1970-P TaqMan PCR for *M. chimaera*.** (A) Standard curve showing Ct
487 versus *M. chimaera* 10-fold serial dilutions, expressed as genome equivalents (log scale). Shown are
488 the mean and standard deviation for triplicate DNA preparations for assay 1970-P. A curve was
489 interpolated using linear regression, R^2= 0.992. Dotted lines indicate 95% confidence intervals. (B)
490 Detection sensitivity of 1970-P for detection of *M. chimaera* spiked into whole blood, indicating a limit
491 of detection of 10 CFU. Shown are the mean and standard deviation for triplicate blood samples and
492 DNA extractions for each dilution.
493

Tables:

Table 1: Summary of the six putative *M. chimaera*-specific DNA segments identified by comparative genomics

Region ID	Position in ANZ045 chromosome	Length (bp)	Putative CDS spanning region	TaqMan probe ID[1]
SR1	2047981-2052621	4,641	2 x hypothetical proteins	1970
SR2	2168889-2169420	531	1 x hypothetical protein	
SR3	2171047-2171975	928	1 x hypothetical protein	
SR4	2174560-2175316	756	No protein-coding region detected	
SR5	4855157-4855879	722	1 x hypothetical protein	
SR6	5338898-5339612	714	tRNA-His(gtg)	

Notes: [1]Probe ID number is based on the position of the first nucleotide of the TaqMan probe in the DNA segment (refer Table 2)

Table 2: Sequences of the *M. chimaera*-specific TaqMan primers and probe

	ID	Sequence (5'-3')	*M. chimaera*-specific region	Position in ANZ045 chromosome	Amplicon length (bp)
TaqMan Probe	1970-P	ACTCAAACACCTGACGAGTCA	SR1	2,049,950-2,049,970	79
Forward primer	1939-F	ACTTGACGAGGTCTTGCAGG	SR1	2,049,919-2,049,938	
Reverse primer	2017-R	GACGGCATAGAGATTCGCCA	SR1	2,049,978-2,049,997	

505 **Table 3: Environmental sampling qPCR, culture and heterotrophic plate count summary**

SAMPLE ID	1970-P (Ct)	Genome equivalents	Volume water filtered (mL) (qPCR)	Genome equivalents/mL water	Culture positive (50 mL)	HCC/mL (log_{10})	Source
17444	No Ct	ND	500	ND	no	2.7	Facility C: HCU
17651	No Ct	ND	200	ND	no	3.2	Facility F: HCU
17851	No Ct	ND	216	ND	no	ND	Facility D: HCU cardioplegia tank
18078	No Ct	ND	420	ND	no	ND	Facility G: HCU cardioplegia tank
18298	No Ct	ND	480	ND	no	ND	Facility G: HCU cardioplegia tank
18849	No Ct	ND	220	ND	no	ND	Facility G: HCU cardioplegia tank
18850	No Ct	ND	130	ND	no	ND	Facility G: HCU cardioplegia tank
19130	No Ct	ND	420	ND	no	1	Facility D: HCU
19330	37.76	40	1000	2	no	ND	Facility G: HCU cardioplegia tank
17443	37.67	42	34	62	no	2.8	Facility C: HCU
14211	36.17	98	270	18	yes	ND	Facility C: HCU
18079	36.04	105	960	5	no	ND	Facility G: HCU cardioplegia tank
17164	35.22	167	60	139	no	3.1	Facility E: HCU
9221	34.32	277	60	231	yes	ND*	Facility A: HCU overflow water bottle
18297	34.32	277	1000	14	no	ND	Facility G: HCU cardioplegia tank
18080	33.79	372	420	44	no	1	Facility G: HCU cardioplegia tank
10895	32.96	593	50	593	yes	ND	Facility B: HCU unit-3 Patient circuit
10896	32.65	706	70	504	yes	ND	Facility B: HCU unit-3 cardioplegia circuit
19128	32.53	755	420	90	no	ND	Facility D: HCU
10892	32.26	879	50	879	yes	ND	Facility B: HCU unit-1 cardioplegia circuit
10894	31.85	1106	30	1843	yes	ND	Facility B: HCU unit-2 cardioplegia circuit
18081	30.46	2412	960	126	no	1	Facility G: HCU cardioplegia tank
17850	30.19	2806	300	468	no	1	Facility D: HCU cardioplegia tank
19129	30.13	2902	400	363	no	1	Facility D: HCU
16569	29.19	4917	200	1229	no	4.3	Facility D: HCU unit-1
18949	29.13	5086	220	1156	no	ND	Facility H: HCU cardioplegia circuit

18948	28.69	6509	275	1183	no	ND	Facility H: HCU patient circuit
18951	28.58	6923	280	1236	no	ND	Facility H: HCU cardioplegia circuit
10897	25.98	29765	70	21261	yes	1	Facility B: HCU unit-1 patient circuit
16571	24.82	57055	200	14264	yes[#]	1	Facility D: HCU unit-3
10893	23.55	116325	80	72703	yes	2.7	Facility B: HCU unit-2 Patient circuit
18950	23.04	154851	130	59558	no	ND	Facility H: HCU patient circuit
16570	21.31	408655	200	102164	yes	1	Facility D: HCU unit-2

506 **Notes:** [#]culture contaminated; * ND = not detected

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510 **Table 4: Correspondence between *M. chimaera* culture and 1970-P qPCR in HCU samples**

	Culture positive	Culture negative	Total
qPCR +	10	15	25
qPCR -	0	8	8
Total	10	23	33

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