Metal worker’s lung; spatial association with *Mycobacterium avium*

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Online Data Supplement

Supplementary Figure

Figure S1. Boxplots showing the mycobacterial and total bacterial burden in workstations which are A) sump-fed or standalone, and B) associated with the outbreak zone or not. Welchs' two sample t-test results are annotated on the plot and values for the factory sump are added for clarity. Horizontal black lines represent the median value while the extent of the boxes represents the 25th and 75th percentiles. Vertical lines represent the extent of the data, and points the statistical outliers defined as 1.5xIQR; notches represent the 95% confidence intervals.

Table S1. Accession numbers for HSP65 genes used for the construction of the phylogenetic tree in Figure 3.

Taxa Name	Accession number
M. avium	DQ284768.1
M. arosiense	GQ153297.1
M. Intracellulare	GQ153290.1
M. scrofulaceum	AF434733.1
M. Immunogenum	DQ288262.1
M. parascrofulaceum	HM229795.1
M. nebraskense	GQ153294.1
M. malmoense	GQ153293.1
M. Simae	AF434730.1
M. triplex	GQ153291.1
M. paraffinicum	GQ153282.1
M. pseudoshottsii	DQ987722.1
M. chelonae	AF547818.1
M. kansasii	CP006835.1
M. neoaurum	JF491302.1
M. abcessus	KT185531.1
M. terrae	AF434736.1
M. nonchromogenicum	AF434732.1
M. bovis	KJ534361.1
M. gastri	JF491315.1
M. africanum	FJ617583.1
M. fortuitum	AF547832.1
M. vaccae	JF491312.1
M. tuburculosis	JX294381.1
M. triviale	AF434737.1
M. szulgai	JF491308.1
M. tokaiense	JF491309.1
M. smegmatis	HM454229.1
M. shimoidei	AF547874.1
M. interjectum	JF491298.1
M. fortuitum	DQ866789.1

Supplementary Methods

Enumeration of HSP65 gene copy numbers by QPCR

The primers TB11 5'-ACC AAC GAT GGT GTG TCC AT-3' and TB12 5'-CTT GTC GAA CCG CAT ACC CT-3'[28] were used to amplify a 401 bp, phylogenetically discriminative, region of the HSP65 gene belonging to mycobacteria-like organisms. QPCR reactions were performed in triplicate on a Viia 7 real time PCR system (Applied Biosystems, Paisley, UK) in reactions consisting of 7.5 µl Low Rox syber green master mix (Kappa Bioscience, Oslo, Norway), 0.2 µM of each primer, 5 µl of 1 in 5 diluted template and made up to 15 µl with ultra-pure, microbiologically clean certified water (Cambio, Cambridge, UK). Cycling conditions consisted of an initial denaturation step of 95 °C for 3 minutes followed by 40 cycles of 95 °C for 20 second, 60 °C for 30 seconds and 72 °C for 30 seconds.

QPCR standards were generated from a cloned HSP65 gene belonging to the *M. psychrotolerans* type strain (DSM 44697) in PCR-4 vector (Life Technologies Ltd, Paisley, UK). Plasmids were quantified using Quantit picogreen dsDNA Assay kit (Promega, Madison, USA) and serially diluted 10 fold to form standards ranging from 1 x 10^8 1 x 10^3. Triplicate no template controls were also included with 5 µl of microbiologically clean ultrapure water (Cambio, Cambridge, U.K.). Resultant quantities were normalised to represent copy number per ml of MWF.

Enumeration of 16S rRNA gene copy numbers by QPCR

The primers 520F 5'-AYT GGG YDT AAA GNG-3' and 802R 5'-TAC NVG GGT ATC TAA TCC-3' targeting the 16S rRNA gene V4 region were used to enumerate the 16S rRNA gene copy number per ml of MWF. Using the same DNA extracts as the mycobacterial QPCR analysis, reactions were carried out in triplicate on a Viia 7 realtime PCR system (Applied Biosystems, Paisley, UK). Each 15 µl reaction contained 5 µl of a 1 in 5 diluted DNA extract, 0.3 µM of each primer, 7.5 µl Low Rox syber green master mix (Kappa Bioscience, Oslo, Norway) and made up to 15 µl with ultra pure microbiologically clean certified water (Cambio, Cambridge, UK). QPCR standards created from a near full length 16S rRNA gene obtained from *Vibrio natriegens* (DSMZ 759). The 16S rRNA amplicon (positions 27 to 1492 of the Escherichia coli reference) was cloned using TOPO TA cloning kit and 10 fold serial dilutions of the purified plasmid (1x10^8 1x10^4 copies per reaction) were used as standards. Cycling conditions consisted of an initial denaturation step of 95 °C for 3 minutes followed by 40 cycles of 95 °C

for 20 second, 50 °C for 30 seconds and 72 °C for 30 seconds.

Phylogenetic Analysis

Gel purified (Qiagen, Venlo, Netherlands) end point PCR products were generated using 12.5 µl of 2 x Q5 high fidelity mastermix (NEB, Hitchin, U.K.) and 0.2 µM of each primer with 1 µl of template in a 25 µl reaction with ultra pure, microbiologically clean water (Cambio, Cambridge, U.K.) as a dilutant. PCR reactions were carried out over 35 cycles using the previously mentioned themo-cycling protocol. Bidirectional Sanger sequencing was carried out on each positive sample using the primers TB11 and TB12 (Eurofins Genomics, Ebersberg, Germany) and the returned sequences were quality trimmed (< Q30) and manually assembled using Bioedit [29]. In order to assign a likely taxonomy to the generated sequences, a bootstrapped (n = 500) maximum likelihood tree was generated in Mega6[30] using MWF sequences alongside sequences from type strain mycobacterial organisms (Table S1). An out-group sequence from the closely related Nocardia genus was used to root the tree.

Supplementary References

28. Telenti A, Imboden P, Marchesi F, et. al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet. 1993;341(8846):647–51.

29. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–98.

30. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Bio Evol 2013:mst197.