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Accurate subspecies-level identification of clinically significant *Mycobacterium avium* and *Mycobacterium intracellulare* by whole-genome sequencing

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ABSTRACT

Whole genome sequencing (WGS) of *Mycobacterium avium complex* (MAC) isolates in the clinical laboratory setting allows for rapid and reliable subspecies identification of a closely related complex of human pathogens. We developed a bioinformatics pipeline for accurate subspecies identification and tested 74 clinical MAC isolates from various anatomical sites. We demonstrate that reliable subspecies level identification of these common and clinically significant MAC isolates, including *M. avium* subsp. *hominissuis* (most dominant in causing lower respiratory tract infections in our cohort), *M. avium* subsp. *avium*, *M. intracellulare* subsp. *intracellulare*, and *M. intracellulare* subsp. *chimaera*, can be achieved by analysis of only two marker genes (*rpoB* and *groEL/hsp65*). We then explored the relationship between these subspecies and anatomical site of infection. Further, we conducted an *in silico* analysis and showed our algorithm also performed well for *M. avium* subsp. *paratuberculosis* but failed to consistently identify *M. avium* subsp. *silvaticum* and *M. intracellulare* subsp. *yongonense*, likely due to a lack of available reference genome sequences; all the 3 subspecies were not found in our clinical isolates and rarely reported to cause human infections. Accurate MAC subspecies identification may provide the tool and opportunity for better understanding of the disease-subspecies dynamics in MAC infections.

1. Introduction

Mycobacterium avium complex (MAC) is the most frequently isolated nontuberculous mycobacterium (NTM) in humans, particularly in those with HIV infection or cystic fibrosis (Floto et al., 2016; Horsburgh and Selik, 1989). In adults, MAC infection most often manifests as a chronic lung or soft tissue infection (Franco-Paredes et al., 2018). It is also one of the most common causes of subacute or chronic cervical adenitis in children <5 years of age (Wolinsky, 1995; Tebruegge et al., 2016). MAC consists of 12 closely related species, with *M. avium* and *M. intracellulare* being the most frequently isolated (van Ingen et al., 2018). Laboratory diagnosis of MAC is challenging, as it is slow growing and difficult to culture, and clinical importance may be confounded by transient colonization (Busatto et al., 2019). If successfully cultured in the laboratory, the performance of the current gold-standard drug susceptibility test (DST) may not be consistently reliable, and its turn-around-time is mostly too long to meet the clinical needs (Heifets, 1996). Antimicrobial resistance (AMR) is common and associated with poor treatment outcomes that are modulated by the baseline antimicrobial susceptibility of the infecting strain (Cho et al., 2018). Together, limitations in MAC subspecies identification and AMR testing often leaves clinicians with little actionable information on which to base treatment decisions (Koh et al., 2012; Lange et al., 2022; Daley et al., 2020).

To address the many unmet needs in MAC management, we developed a whole-genome sequencing (WGS) assay for MAC species identification and subspecies identification of *M. avium* and *M. intracellulare*. While other molecular methods such as qPCR or MALDI-TOF are less expensive and commonly used in the clinical laboratory setting, they lack sufficient resolution to discriminate between closely related species/subspecies. In terms of sequencing, 16S rRNA gene is the most

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studied genetic marker for bacterial identification and is generally the first choice for sequence analysis. However, it has high similarity in *Mycobacterium* species, and does not provide sufficient resolution to distinguish closely related species (Andreas et al., 1998). Instead, the genes of beta subunit of RNA polymerase (RpoB) and GroEL, also known as Hsp65, a member of the chaperonin protein family, are found to be highly reliable in *Mycobacterium* species identification (Mizzi et al., 2022; Rindi and Garzelli, 2014; Huang et al., 2012; Wong et al., 2003; Telenti et al., 1993).

In this study, we developed an algorithm mainly based on the *rpoB* and *hsp65* (also known as *groEL*) genes for the species/subspecies identification by leveraging standard WGS library preparation methods, a web tool for *k-mer* based identification (Center for Genomic Epidemiology, Lyngby, Denmark), and BLAST (NCBI, Bethesda MD, USA) to perform analysis of consensus gene sequences. The critical step involves BLAST analysis of the full-length *rpoB* and *groEL* consensus sequences. Notably, there are 2 copies of *groEL* gene in each MAC genome. We observe that combining analysis of *rpoB* and *groEL* genes allows us to reliably identify MAC species and the subspecies of *M. avium* and *M. intracellulare*, and even detect mixed populations.

2. Materials and methods

2.1. Ethics

This study was reviewed by the UCLA Human Research Protection Program and received an IRB exemption. All the patients' information was de-identified.

2.2. Sample processing and culture

Specimens (10 mL) were first treated by adding 12.5 mL freshly prepared N-Acetyl-L-Cysteine Sodium Hydroxide (NALC-NaOH) solution for 15 min to remove the normal flora and liquefy the sample; 22.5 mL of phosphate buffer is then added followed by centrifugation at 3600 *g* for 15 min. After the supernatant was decanted, the sediment was resuspended with 1.6 mL sterile Phosphate buffer. A portion of the well suspended sediment (0.5 mL) was then inoculated into BACTEC™ MGIT™ liquid media (Becton Dickenson), which has been added with MGIT 960Supplemented (OADC/PANTA) mixture (Becton Dickenson) and incubated in the BACTEC™ MGIT™ System. The sediment was also inoculated directly onto a Middlebrook 7H11/7H11 selective biplate (Hardy Diagnostics) using a disposable inoculating loops (10 μL) and incubated in an incubator at 37 °C with 6–10% CO2. Once mycobacteria grew from either the BACTEC™ MGIT™ liquid media or the Middlebrook 7H11/7H11 selective biplate, Gen-probe test (Hologic) or MALDI-TOF (Biomerieux) was used to identify MAC, respectively.

2.3. Isolate preparation, DNA extraction and WGS

The MAC isolates were heat-inactivated (100 °C for 30 min) and an additional bead-beating step was performed for mechanical disruption of the cell wall. The Qiagen (Valencia, CA) EZ1 Blood and Tissue Kit and the EZ1 Advanced XL instrument were used according to the manufacturer's instructions to extract genomic DNA from pure microbial isolates. Extracted DNA was quantified with the Qubit 1× double-stranded DNA HS assay using the Qubit 3.0 Fluorometer (Thermo Fisher, Waltham, MA). Acceptable quantities of DNA were ≥ 0.04 ng/μL. The DNA library was prepared using the Illumina DNA Library Prep kit and WGS was performed on the Illumina MiSeq using the 2 × 250 kit (Illumina, San Diego, CA).

2.4. Bioinformatics for subspecies identification

The raw sequence reads are first uploaded to KmerFinder tool from Center for Genomic Epidemiology. Raw reads are then trimmed and mapped to the reference genome for the top KmerFinder hit in CLC Bio (Qiagen). There are 2 copies of *groEL* gene (gene length of 1617 bp and 1626 bp) in each MAC genome, with about 71.0% - 72.4% homology between them (data not shown). Each copy is designated as *groEL1* or *groEL2* to differentiate them. The consensus sequence for *rpoB* and *groEL* (both *groEL1* and *groEL2*) genes are extracted and uploaded to BLAST (NCBI, Bethesda, MD). The *rpoB* gene is used as the primary marker and *groEL* as the secondary marker (Fig. 1). All subspecies with percent identity >99% and percent coverage >99% in the *rpoB* gene are recorded. If no subspecies meet the >99% identity and > 99% coverage quality control criteria, no subspecies identification is made, and the isolate is flagged for investigation of mixed subspecies. If only one subspecies meets the criteria, that result is accepted as subspecies identification. If more than one subspecies meets the criteria, the *rpoB* and *groEL* (either copy) top hits, as defined by the BLAST results with the highest percent identity with coverage >99%, are compared. If the *groEL* (either copy) top hit matches the *rpoB* top hit, the result is accepted as the subspecies identification. In rare cases there may be multiple subspecies tied for the *groEL* top hit. In this case, if the subspecies identified by the *rpoB* top hit is among those tied for the *groEL* top hits, the results are congruent and therefore the *rpoB* top hit is accepted as the final identification. If *rpoB* and *groEL* results are incongruent, a SNP tree analysis is performed using the Basic Variant Detection tool in CLC Bio Genomics Workbench, which generates variant tracks following read mapping to the reference genome. The variant tracks for each isolate are then compared using the Create SNP Tree tool. SNP trees are created using maximum-likelihood algorithms. The subspecies of an isolate is determined by the subspecies of other samples clustered together in the SNP tree.

2.5. Average nucleotide identity (ANI) analysis

ANI analysis was conducted to confirm WGS subspecies identification by using the CLC Bio's built-in ANI tool with the default settings. Reads were trimmed and mapped to the reference genome for each subspecies for which a complete reference genome assembly was available. A consensus sequence was extracted from mapped reads and a whole genome alignment was created from consensus sequences. ANI analysis was performed from the whole genome alignment. Subspecies identification was considered confirmed if the ANI was calculated to be >98%. Reference genomes used as type strain include *M. avium* subsp. *hominissuis*: *M. avium* 104 (NCBI: GCF_000014985.1) (van Ingen et al., 2018; Turenne et al., 2007; Turenne et al., 2006); *M. avium* subsp. *avium*: *M. avium subsp. avium* DSM44156 (NCBI: GCF_009741445.1); *M. intracellulare* subsp. *intracellulare*: *M. intracellulare subsp. intracellulare* (FDAARGOS_1610: GCF_021183785.1); *M. intracellulare* subsp. *chimaera*: *M. intracellulare subsp. chimaera* (NCBI: GCF_002101575.1); *M. intracellulare* subsp. *yongonense*: *M. intracellulare subsp. yongonense*, (NCBI: NC_021715). Notably, no complete assembly is available for *M avium. Subsp. silvaticum* as of the writing of this manuscript.

3. Results

The clinical isolates came from a total of 65 patients seen within the UCLA Health System located in the greater Los Angeles Metropolitan Area of Southern California. Twenty-five of the patients were immunocompromised including: solid organ transplant recipients ($n = 11$), immunosuppressive therapy recipients for an autoimmune condition ($n = 6$), primary/genetic immunodeficiency (including *CF*, $n = 5$) and HIV ($n = 3$).

To test our algorithm (Fig. 1), we analyzed 74 clinical MAC isolates collected by the UCLA Clinical Microbiology Lab between 2018 and 2022. Using WGS, we provide a single subspecies identification for 72/74 (97.3%) isolates and identify mixed dual subspecies in 2/74 (2.7%) of isolates. One sample (UCLA_285), was found to have incongruent *rpoB* and *groEL* subspecies identification results. For this sample, the

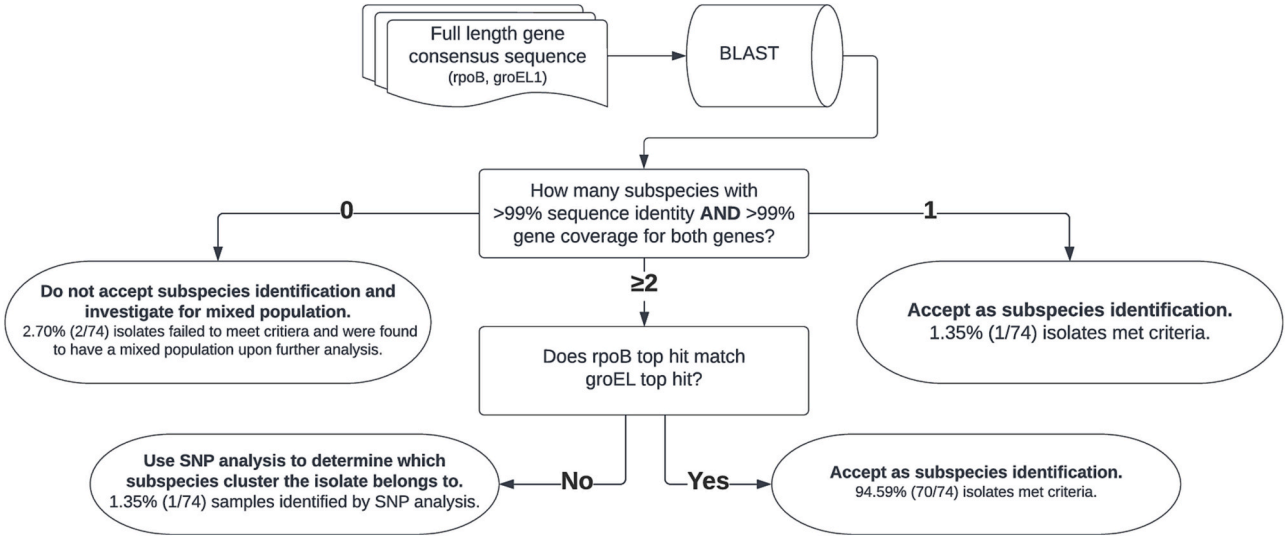

Fig. 1. Workflow diagram of algorithm to determine subspecies identification from full-length consensus gene sequences.

subspecies was successfully identified using single nucleotide polymorphism (SNP) tree analysis (Fig. 2). SNP tree analysis further confirmed the accuracy of the marker gene-based results by showing a clear separation of different subspecies within *M. avium* (Fig. 2) and *M. intracellulare* (Fig. 3).

We also performed ANI analysis to further confirm the subspecies identification of our study samples. The ANI analysis showed >98% score in all the subspecies identified in this study: *M. avium* subsp. *hominissuis* (98.17–100%, $n = 41$), *M. avium* subsp. *avium* (98.73–99.98%, $n = 8$), *M. intracellulare* subsp. *intracellulare* (98.95–100%, $n = 13$), and *M. intracellulare* subsp. *chimaera* (98.26–100%, n = 8) (Supplemental Figs. S1–4). Since we did not identify any *M. avium* subsp. *paratuberculosis, M. avium* subsp. *silvaticum, and M. intracellulare* subsp. *yongonense* in our study samples, we downloaded available high-quality reference genomes belonging to each subspecies (Supplemental Table S1) and performed *in silico* analysis. Our algorithm demonstrated perfect performance for *M. avium subsp. paratuberculosis* (100%, 10/10), but suboptimal performance for *M. avium subsp. silvaticum* (0%, 0/1, misidentified as *M. intracellulare* subsp. *intracellulare*), *and M. intracellulare subsp. yongonense* (50%, 3/6, the other 3 samples were misidentified as *M. intracellulare* subsp. *chimaera* or discrepant between the results from *rpoB and groEL*) (Table S1).

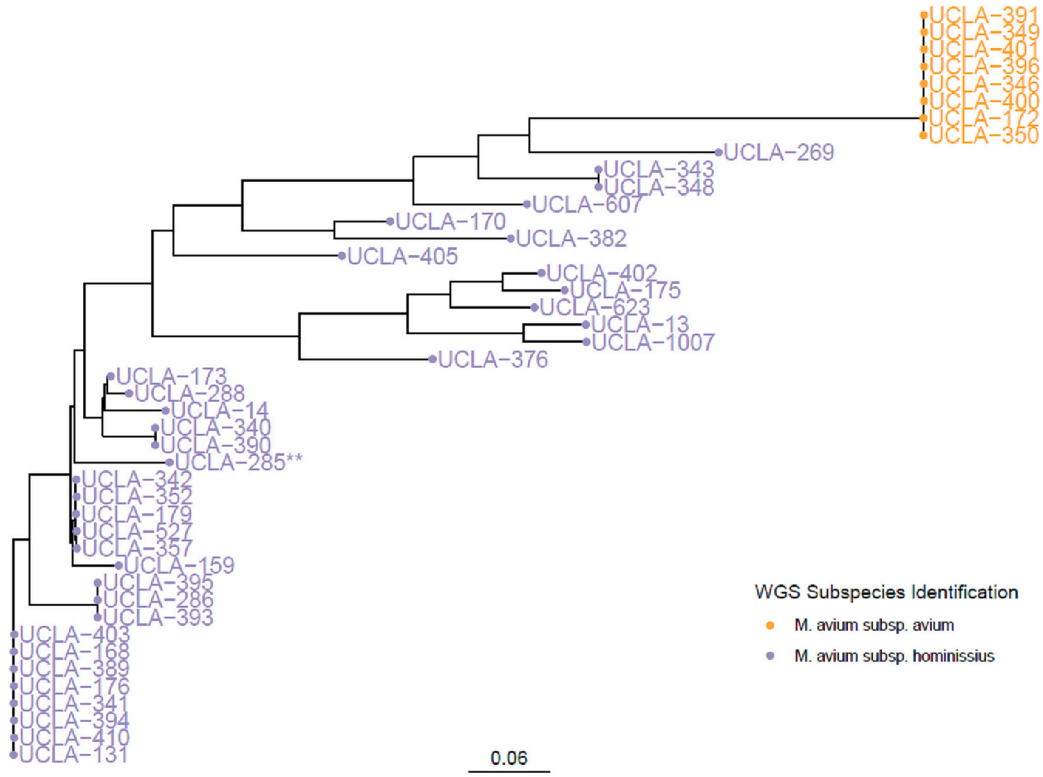

Fig. 2. SNP tree of *M. avium* subspecies.
** = discordant *rpoB, groEL* identification results.
The unit of the scale bar is nucleotide substitutions per site.

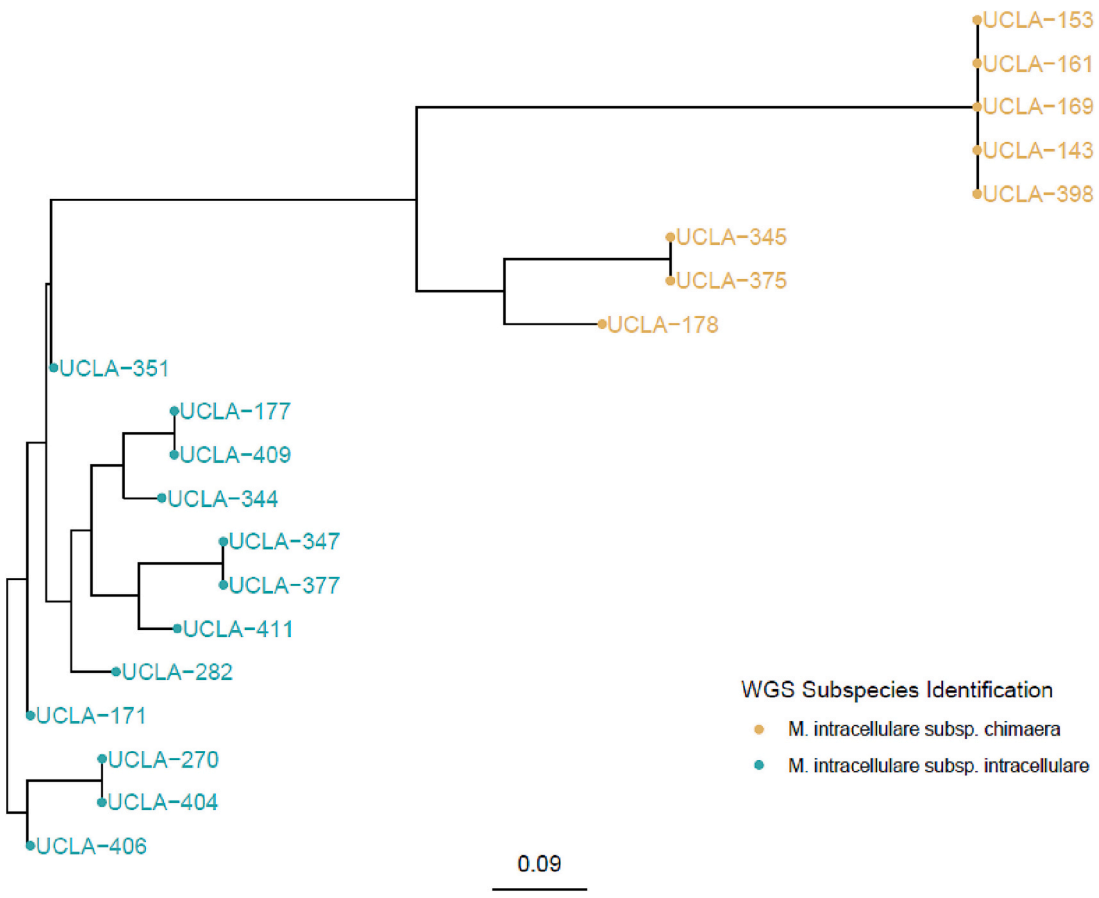

Fig. 3. SNP tree of *M. intracellulare* subspecies.
The unit of the scale bar is nucleotide substitutions per site.

By combining clinical information with WGS subspecies identification, we show that in our patient cohort, *M. avium* subsp. *hominissuis* (most dominant, 60%, 33/56), *M. intracellulare subsp. intracellulare* (20%, 11/56) and *M. avium* subsp. *avium* (13%, 7/56) are the most frequently identified subspecies in lower respiratory tract (LRT) infections while *M. intracellulare subsp. chimaera* was mainly isolated from wound infections (Fig. 4). Various MAC subspecies were also identified in isolates from stool, bone, blood, and peritoneal fluid. Mixed MAC infections were only identified in LRT specimens. Notably, *M. avium subsp. hominissuis* and *Mycobacterium colombiense* were the only species identified from blood cultures.

4. Discussion

In this study we developed and employed a bioinformatics pipeline for MAC subspecies identification. We tested these methods in a cohort of clinical samples from a single center and report results alongside clinical data correlates, uncovering patterns in infection site distribution. Our study reveals that analysis of just two genes, *rpoB* and *groEL*, provides sufficient discriminatory power for accurate MAC subspecies identification in most samples. We also found LRT infections are most often due to *M. avium* subsp. *hominissuis* in our patient population, however, further studies are needed to understand the clinical and epidemiological implication of this phenomenon.

As reliable subspecies-level identification in MAC has been relatively unavailable to date, the clinical relevance of this data is yet to be characterized. However, a recent study found significant differences in drug susceptibility patterns among MAC species/subspecies (Fernandez-Pittol et al., 2023), highlighting the potential ability of subspecies-level analysis to yield valuable clinical insights. We argue that the increased accuracy of WGS-based subspecies identification should be leveraged in further studies looking to determine the clinical characteristics of MAC infections. Furthermore, subspecies-stratified AMR genotypic-phenotypic correlation analysis may represent a promising path to more reliable prediction of drug susceptibility in MAC infections.

Beyond subspecies identification, the routine use of WGS in the clinical setting provides the opportunity to impact both longitudinal patient monitoring and population-based interventions. As patients with MAC disease and underlying conditions, such as cystic fibrosis or HIV, are regularly sampled, longitudinal analysis of isolates from the same patient can help distinguish a treatment-resistant relapse from reinfection, as is currently done with other mycobacteria (Liu et al., 2022; Stefani et al., 2017; Chawla et al., 2022). Additionally, WGS data can be employed by infection control and epidemiological investigation teams to provide genomic evidence of transmission events (Xu et al., 2022; Greninger and Zerr, 2021).

The main limitation of the algorithm developed in this study is that it does not perform well on the identification of 2 MAC subspecies: *M. avium* subsp. *silvaticum* and *M. intracellulare* subsp. *yongonense*, both of which were not found in our clinical isolates and rarely reported to cause human infections, especially in North America. The reason for this discrepancy is unclear but may be attributed to several factors. First, the key step in our algorithm, searching the NCBI database, is dependent upon the presence of high-quality annotated reference genomes that accurately represent the genomic diversity of MAC subspecies. For instance, only one fully assembled reference genome is available for *M. avium subsp. silvaticum*. Similarly, very few high-quality reference genomes are available for *M. intracellulare* subsp. *yongonense*. Our failure to accurately identify these subspecies using BLAST search indicates that without an adequate representation of high-quality annotated reference

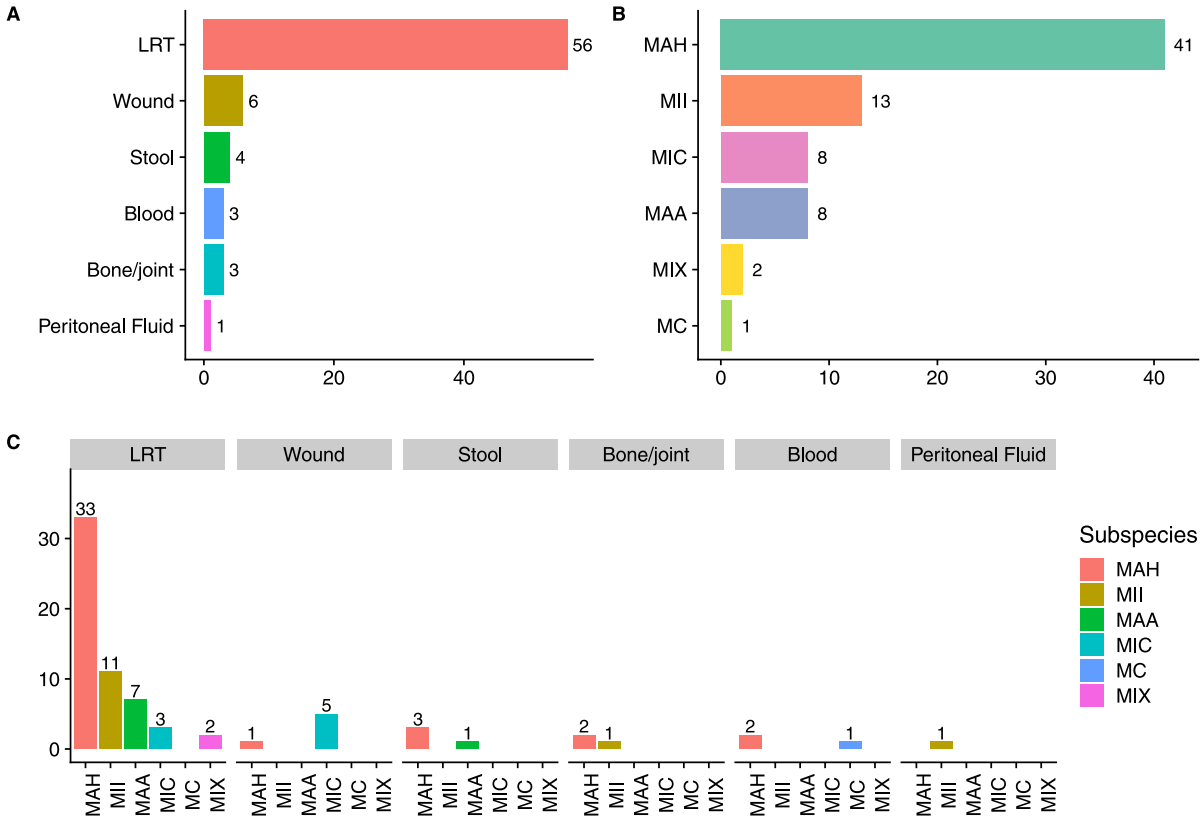

Fig. 4. WGS ID reveals clinically important patterns in MAC infection. A) Body site of isolation for MAC clinical isolates. B) Distribution of WGS-identified subspecies. C) Subspecies identification patterns across clinical isolate body site. LRT = lower respiratory tract, MAH = *M. avium. subsp. hominissuis,* MII = *M. intracellulare* subsp. *intracellulare,* MIC = *M. intracellulare susbp. chimaera,* MAA = *M. avium* subsp. *avium,* MIX = mixed subspecies, MC = *M. colombiense*.

genomes present in the BLAST database, the performance of our algorithm suffers. Second, the recent taxonomic re-classification of *M. intracellulare subsp. yongonense* may be problematic due to its inherent genetic diversity (Castejon et al., 2018). These challenges are well known for MAC as a unique *Mycobacterium* group that's highly genetically similar but extremely heterogeneous presenting with a wide spectrum of ecological niches and behaviors (Mizzi et al., 2022; Rindi and Garzelli, 2014). The continuous taxonomic reclassification of MAC is expected and therefore the algorithm developed in this study will need to undergo update/modification as future taxonomic changes happen.

In summary, we show that a simple algorithm analyzing just two genes provides highly reliable subspecies identification among previously difficult to distinguish microbial pathogens. We validate our algorithm in a cohort of clinical samples, illustrating the power of WGS to enable more accurate diagnosis of MAC infections. Ultimately, we believe that the use of WGS will impact patient care beyond laboratory diagnosis by creating the opportunity for better understanding of the disease-subspecies dynamics in MAC infections.

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Declaration of Competing Interest

There are no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2023.106726.

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