Mycobacterium abscessus infection in cystic fibrosis: molecular typing and clinical outcomes

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Mycobacterium abscessus is a significant pathogen in the cystic fibrosis patient population. PCR amplification and sequencing can provide accurate subspecies identification, and can predict macrolide susceptibility, which is becoming increasingly important for patient management. Molecular techniques for further typing of isolates provide tools for the ongoing investigations into the clinical impact of particular M. abscessus strains. Whole-genome sequencing is likely to be the only technique that provides sufficient resolution for investigating transmission events between patients.

Mycobacterium abscessus infection in cystic fibrosis (CF)

Rapidly growing mycobacteria (RGM) are non-tuberculous mycobacteria (NTM) that infrequently cause disease in healthy people and are commonly environmental microorganisms found in soil, bioaerosols and natural and chlorinated water. They grow in the laboratory on solid media, such as blood or chocolate agar, in 3–7 days. Colonies can have either a rough or smooth morphotype. The most notable RGM species are Mycobacterium abscessus, Mycobacterium chelonae, Mycobacterium fortuitum and Mycobacterium mucogenicum, all of which cause respiratory and soft tissue infections in humans. The source of these infections is still uncertain, but tap water and person-to-person transmission have both been suggested (Saiman & Siegel, 2004; Feazel et al., 2009; Bryant et al., 2013; Thomson et al., 2013).

M. abscessus is a rapidly growing, multidrug-resistant organism that has emerged as a significant pathogen in CF patients. Estimated to have an overall prevalence rate in this patient group of up to 13% in Europe and 16% in the USA, M. abscessus accounts for over half of all NTM isolated (CFF, 2012; Seddon et al., 2013). The rising prevalence of infection with M. abscessus in CF patients could be partly attributable to a number of factors, including improved laboratory practice, improved patient survival, increased environmental exposure (Feazel et al., 2009; Thomson et al., 2013), inhaled antibiotic usage (Saiman & Siegel, 2004) and person-to-person spread (Bryant et al., 2013). M. abscessus has been linked to poor clinical outcomes in the CF population, especially following lung transplantation, with many centres not listing infected patients for transplant assessment (Gilljam et al., 2010; Luong et al., 2010; Watkins & Lemonovich, 2012).

M. abscessus was first described as a species in 1992, having been previously considered a subspecies of M. chelonae (Kusunoki & Ezaki, et al. 1992). Mycobacterium bolletii and Mycobacterium massiliense were recognized as further new NTM species in 2006 (Adékambi & Drancourt, 2004; Adékambi et al., 2006; Euzéby, 2006). Collectively these three species are sometimes referred to as the M. abscessus complex. However, there has been some debate in the literature regarding the taxonomy of these organisms, which cannot be differentiated by phenotypic methods. Genotypic studies have led to the proposal that the three taxa should be combined into a single species, and that M. massiliense and M. bolletii should be further combined as a single subspecies (M. abscessus subsp. bolletii), while M. abscessus remains as a separate subspecies (Leao et al., 2009, 2011). However, recent higher-resolution typing, including whole-genome sequencing, appears to show that M. abscessus, M. massiliense and M. bolletii exist as three separate entities and supports the designation of three subspecies (Harris et al., 2012; Bryant et al., 2013). In the context of this review, we shall refer to M. abscessus subsp. abscessus, M. abscessus subsp. massiliense and M. abscessus subsp. bolletii for the sake of clarity, as this is consistent with the majority of the published studies that will be discussed here.

Current treatment strategies for M. abscessus infection in CF patients

M. abscessus, in common with other mycobacterial species, has high levels of natural resistance to most classes of antibiotics, due to the presence of an impermeable cell wall, antibiotic-modifying/inactivating enzymes, efflux pumps and genetic polymorphisms in target genes. Additionally, M. abscessus is resistant to classical antituberculous drugs.
such as rifampicin and ethambutol (Nessar et al., 2012). Treatment guidelines issued by the American Thoracic Society in 2007 recommended a multi-drug regimen that included a macrolide (clarithromycin) for the treatment of pulmonary *M. abscessus* disease (Griffith et al., 2007). More recent guidelines for the management of *M. abscessus* infection in CF patients recommended treatment with a macrolide (azithromycin), an aminoglycoside (amikacin) and at least one other drug of a different class (Leung & Olivier, 2013).

Resistance to macrolides in *M. abscessus* can occur due to post-transcriptional methylation of the 23S rRNA molecule. This can be high-level resistance that is acquired due to mutations in the *rrl* gene that encodes the 23S rRNA and in *M. abscessus* resistance can also be due to the presence of an inducible ribomethylase gene, *erm*(41) (Nash et al., 2009). The key difference in antimicrobial susceptibility between the three *M. abscessus* subspecies is expression of the *erm*(41) gene. A truncated and non-functional *erm*(41) gene occurs in *M. abscessus* subsp. *massiliense* and this subspecies typically remains susceptible to clarithromycin, although resistance may occur if strains acquire mutations in the *rrl* gene. Additionally, a point mutation (T28C) in *erm*(41) confers clarithromycin susceptibility to some *M. abscessus* subsp. *abscessus* strains (Bastian et al., 2011). Susceptibility to macrolides is routinely assessed using microdilution methods, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011) (formerly known as the National Committee for Clinical Laboratory Standards). Phenotypic macrolide resistance in *M. abscessus* could be inferred by sequencing the *erm*(41) and *rrl* genes (Bastian et al., 2011), but this is not currently implemented in routine practice.

**Identification of *M. abscessus* isolates**

Identification of mycobacterial species is usually performed using genotypic methods. Two commercial line probe assays are employed by a number of laboratories, INNO-LIPA MYCOBACTERIA v2 (Innogenetics) and the Genotype Mycobacterium CM kit (Hain Lifescience). Both assays can accurately identify the majority of commonly encountered mycobacterial species, but the Genotype Mycobacterium CM kit may sometimes have difficulty differentiating *M. chelonae* from *M. abscessus*. Neither assay can differentiate between the three *M. abscessus* subspecies (Scarpato et al., 2001). This lack of accuracy in identification is concerning because it may impact on patient management, in particular the decision to list patients for lung transplantation, because *M. abscessus* has been linked with poorer clinical outcomes than those observed in patients infected with *M. chelonae* (Chalermskulrat et al., 2006; Broda et al., 2013). More accurate identification can be performed by PCR and sequencing of housekeeping genes targets such as *hsps5, rpoB, sodA* and *secA* (Ringuet et al., 1999; Adékambi & Drancourt, 2004; Arnold et al., 2012; Blauwendraat et al., 2012). While most isolates can be identified with two targets, multilocus sequence analysis (MLSA) is required to differentiate the three *M. abscessus* subspecies reliably, particularly as some isolates give ambiguous results with single or dual gene targets (Macheras et al., 2009). Fig. 1 illustrates this for five example isolates. A number of reports in the literature describe ambiguous sequencing results from some isolates that have chimeric housekeeping gene sequences, presumably as a result of horizontal gene transfer and recombination (Zelazny et al., 2009; Macheras et al., 2011), which further highlights the challenges of accurate identification and differentiation of these subspecies from one another.

**Molecular characterization of *M. abscessus* complex isolates**

Further differentiation of *M. abscessus* isolates has been achieved using a number of different molecular methods, which are summarized below.

**PFGE**

PFGE allows the separation of large DNA fragments, such as whole or digested chromosomal DNA, by use of a ‘pulsed’ or alternating voltage gradient, which permits much greater resolution than can be achieved by conventional electrophoresis. PFGE has been used to type many bacterial species to strain level, and is routinely used to investigate cross-transmission events and outbreaks (Palazzo et al., 2011; Sabat et al., 2013). However, this technique is largely confined to reference laboratories because it is laborious and technically demanding. Mycobacterial species present a particular challenge because of DNA degradation, which leads to smearing; this may be because the methods used to break the acid-fast cell wall also fragment the genomic DNA. Despite these issues, PFGE has been successfully used to compare isolates of *M. abscessus*, *M. bolletii* and *M. massiliense* in several studies (Jónsson et al., 2007; Matsumoto et al., 2011). PFGE was used alongside MLSA and repetitive sequence-based PCR (rep-PCR) to characterize a large number of non-outbreak *M. abscessus* isolates (Zelazny et al., 2009) and has also been used to investigate a pseudo-outbreak of *M. abscessus* arising from laboratory contamination (Blossom et al., 2008).

**Rep-PCR**

The method uses PCR primers that amplify repetitive sequences in the genome to produce a profile of peaks or bands. A commercial rep-PCR method (Diversilab; bioMérieux) has been employed in a number of studies for typing *M. abscessus* isolates (Harris et al., 2012; Jamal et al., 2014). This relatively simple PCR-based technique can be performed in routine laboratories and the commercial assay includes web-based analysis software.

Rep-PCR has been used to investigate the relationship between *M. abscessus* isolates from a cohort of paediatric CF patients in the UK (Harris et al., 2012) and to investigate an outbreak on a paediatric intensive care unit.
in Kuwait (Jamal et al., 2014). However, it is not yet clear whether identical rep-PCR profiles represent isolates that are the same strain or simply highly related strains of the same genotype.

**Multilocus sequence typing (MLST) and multispacer sequence typing (MST)**

MLST is an extension of the MLSA technique described for the identification of *M. abscessus* isolates to subspecies level (Macheras et al., 2011). However, more housekeeping gene targets are sequenced for MLST (usually around eight). It is robust and reproducible and does not require large amounts of DNA, but the sequences of the housekeeping targets used are very well conserved in this group of organisms and, therefore, MLST does not provide much more discrimination than the identification of the three subspecies (Macheras et al., 2014). However, a recent study described a standardized scheme, which showed good concordance with PFGE (Kim et al., 2013), and this has already proved useful for broad, global comparison of strain types. MST is a further extension of this technique.

Fig. 1. Dendrograms derived from (a) *hsp65*, (b) *rpoB* and (c) concatenated *hsp65* and *rpoB* sequences. Clinical isolates 1–5 are compared to *M. abscessus* complex, *M. chelonae* and *M. fortuitum* reference sequences obtained from GenBank using the CLUSTAL W algorithm in the MEGALIGN program (DNASTAR). Isolates 1–4 can be identified as *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. abscessus* subsp. *bolletii* and *M. chelonae*, respectively, by (a) the *hsp65* target sequence, (b) the *rpoB* target sequence and (c) the concatenated *hsp65/rpoB* target sequences. However, isolate 5 is identified as *M. abscessus* subsp. *abscessus* by the *hsp65* target sequence and *M. abscessus* subsp. *bolletii* by the *rpoB* target sequence, the concatenated *hsp65/rpoB* sequences give an ambiguous result. Further sequencing of other targets (such as *sodA* and *secA*) should be performed on this isolate and the consensus result accepted.
The spacer sequences between 16S and 23S rRNA genes are less well conserved, so this technique may provide greater discrimination. A recent study described the development of a MST scheme for identification of different genotypes of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (Sassi et al., 2013).

**Variable number tandem repeat (VNTR) profiling**

This is a PCR-based method that targets variation in tandem DNA repeats at specified genomic loci. Normally between 9 and 15 loci are chosen, with a numerical profile being generated reflecting the number of repeats at each locus. VNTR analysis is a rapid, portable method for comparing isolates and has been shown to provide equivalent typing resolution to that offered by PFGE, which was formerly the gold-standard for many organisms (Turton et al., 2010). Several recent published studies have described VNTR typing schemes for *M. abscessus* isolates (Harris et al., 2012; Wong et al., 2012; Davidson et al., 2013). One study compared the VNTR scheme to the Diversilab rep-PCR method and characterized *M. abscessus* isolates from a cohort of paediatric CF patients (Harris et al., 2012). The results showed that these patients were infected with isolates that shared a limited number of VNTR profiles. This raises the possibility of cross-transmission or a common environmental source of infection, suggesting further studies focusing on these strains may be merited.

**Whole-genome sequencing**

High-throughput sequencing can obtain sequence data of the entire genome of a number of *M. abscessus* isolates in a single experiment. A recent study used whole-genome sequencing to infer that cross-transmission had occurred in a cohort of adult CF patients (Bryant et al., 2013). Very recently another study (Davidson et al., 2013) demonstrated highly similar genome sequences from *M. abscessus* isolated from different patient populations at different global locations, most notable being near-identical genome sequences from *M. abscessus* from cutaneous infections in Brazilian patients and pulmonary infections in UK CF patients. This highlights the importance of careful analysis for such closely related genome sequences, especially when investigating potential cross-transmission. Additionally whole-genome sequencing has proved invaluable in revealing single nucleotide polymorphisms in virulence factor regulators among serial isolates of *M. abscessus* from CF patients (Kreutzfeldt et al., 2013). The high degree of resolution offered by whole-genome sequencing permits much finer typing than any of the previously described methods and has the capacity to provide valuable information about transmission events.

**Impact of identification and typing on clinical outcomes**

*M. abscessus* has emerged as a significant pathogen in CF patients, linked to disease progression and poor outcomes. Many centres consider *M. abscessus* infection to be a contraindication for lung transplantation. Conversely, patients infected with *M. chelonae* are still considered for transplantation (Gilljam et al., 2010; Luong et al., 2010; Watkins & Lemonovich, 2012). Reports in the literature that relate transplant outcome with the infecting mycobacterial species are likely to be confounded by the inherent inaccuracy in identification methods, with many *M. abscessus* isolates having been incorrectly identified as *M. chelonae*. Furthermore, unless PCR and sequencing techniques have been used, isolates will have been identified only as ‘*M. abscessus*’ and not differentiated further. This may turn out to be a very important requirement if the three *M. abscessus* subspecies are shown in the future to be associated with differing clinical outcomes, particularly following lung transplantation, as recent reports have suggested (Koh et al., 2011; Harada et al., 2012; Robinson et al., 2013). Predicting macrolide susceptibility by sequencing *erm(41)* and *rrl*, alongside accurate identification of the three *M. abscessus* subspecies, would help to inform antimicrobial treatment regimens if performed routinely on *M. abscessus* isolated from CF patients (Bastian et al., 2011; Choi et al., 2012; Nessar et al., 2012; Leung & Olivier, 2013). *M. abscessus* subsp. *abscessus* appears to be linked with worse clinical outcomes than the other subspecies, perhaps due in part to differing macrolide susceptibility (Koh et al., 2011; Harada et al., 2012; Robinson et al., 2013). However, it is important to consider the impact of other factors that may also contribute to clinical outcomes, such as smear positivity and host factors such as CF mutation and immunological status, as these things are also likely to play an important role. Further investigation into how all these factors combine to effect clinical outcomes is now required, and accurate identification of *M. abscessus* isolates will be critical.

In non-CF patient populations there have been several reports of outbreaks of *M. abscessus* subsp. *massiliense* in post-surgical, cutaneous infections (Duarte et al., 2009; Leao et al., 2009; Shang et al., 2011). These outbreaks were all caused by a single strain as determined by PFGE, which was thought to be particularly invasive and pathogenic. Recent studies have suggested that some strains of *M. abscessus* could be associated with chronic infection in a cohort of CF patients (Harris et al., 2012) and that certain strains may also be more transmissible in this patient group (Bryant et al., 2013). It has also been observed that the same strain or genotype is found in a number of CF patients with no apparent epidemiological links (Harris et al., 2012) and that common strains are seen in patients in remote geographical locations (Davidson et al., 2013). These early studies suggest that particular genotypes or strains of *M. abscessus* have differing clinical outcomes or are particularly well adapted to humans. The epidemiology of *M. abscessus* strains in CF and other patient populations warrants further investigation using the molecular methods we have described. However, whole-genome sequencing is the ultimate method for *M. abscessus* typing and, due to the
high level of similarity seen between many M. abscessus strains, this may be required for differentiating closely related strains, particularly when investigating potential cross-transmission events.

References


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