Original Article

Molecular Typing of *Mycobacterium Abscessus* Isolated from Cystic Fibrosis Patients

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Abstract

Background: The possibility of inter-human transmission of *Mycobacterium abscessus* in cystic fibrosis centres has been recently hypothesized suggesting the need for the molecular characterization of strains isolated from such patients. **Materials and Methods:** One hundred and forty one isolates of *M. abscessus* grown from sputum samples of 29 patients with cystic fibrosis were genotyped resorting to variable number of tandem repeats (VNTR) determination and whole genome sequencing (WGS). **Results:** Out of 29 VNTR profiles, 15 were unique to the same number of patients while seven were shared by multiple patients. WGS showed that only two of the patients sharing common VNTR patterns were indeed infected by the same strain. The shared VNTR patterns were mostly present among the isolates of *M. abscessus* subsp. *abscessus*. **Conclusion:** As expected WGS showed a clearly higher discriminatory power in comparison with VNTR and appeared the only molecular epidemiology tool suitable to effectively discriminate the isolates of *M. abscessus* subsp. *abscessus*.

Keywords: Cystic fibrosis, *Mycobacterium abscessus*, VNRT, whole genome sequencing

Introduction

Mycobacterium abscessus is, among the rapidly growing mycobacteria, the species most frequently involved in human infections. In adjunct to the lung, cutis, soft tissues, bone, and joints may be affected.[1] As other nontuberculous mycobacteria, it is present in the environment, is oligotrophic, resistant to chlorination, and is characterized by prolonged survival in biofilms.[2] Its isolation from drinking water has been repeatedly reported.[3,4] Environmental aerosols are considered the major source of contagion for respiratory infections, but the interhuman transmission has been recently hypothesized as well.[5] *M. abscessus* is intrinsically resistant to most antimicrobials, the infections are, therefore, hardly treatable, and the eradication cannot be easily achieved.[6]

Cystic fibrosis (CF) patients are particularly susceptible to pulmonary infections caused by mycobacteria and expecially by *M. abscessus*.[7] In such patients, a prevalence higher than 10% is estimated worldwide.[8] The species is split into three subspecies, i.e., *M. abscessus* subsp. *abscessus* (Masa), *M. abscessus* subsp. *bolletii* (Masb), and *M. abscessus* subsp. *massiliense* (Masm).[9] This taxonomic distinction is also clinically relevant as Masa and Masb have a functional *erm* (41) gene[10] which confers inducible macrolide resistance; this gene is instead truncated in Masm which, consequently, lacks inducible resistance.

In the last years, possible outbreaks of *M. abscessus* in several CF centers have been reported,[5,11] suggesting the need for the molecular characterization of strains isolated from such patients.

Different molecular fingerprinting techniques have been used for *M. abscessus* strains.

Pulsed-field gel electrophoresis of the digested bacterial chromosome yields reproducible patterns of DNA fragments allowing strains differentiation within *M. abscessus* subspecies.[12,13] The technique is, however, prone to DNA degradation leading to uninterpretable results.[14]

Multilocus sequence typing is based on the analysis of polymorphisms in the sequences of selected housekeeping

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Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_33_17

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How to cite this article: Trovato A, Baldan R, Costa D, Simonetti TM, Cirillo DM, Tortoli E. Molecular typing of *Mycobacterium Abscessus* isolated from cystic fibrosis patients. Int J Mycobacteriol 2017;6:138-41.

genes.[15] The discriminative power is proportional to the number of genes investigated and is further increased when the internal transcribed spacer between 16S and 23S is sequenced as well.[16]

In the last years, the analysis of variable number of tandem repeats (VNTR) present at specific loci has been adapted to *M. abscessus*, on the wake of the excellent experience accumulated with *Mycobacterium tuberculosis*. The discriminative potential was at least comparable to that of techniques mentioned above.[17-20]

Very recently, however, high-throughput sequencing has opened up new horizons, and the comparison of whole genomes, unimaginable before, has become reality. It is, therefore, expected that whole genome sequencing (WGS), thanks to its ultimate potential, will soon become the standard for molecular epidemiology investigations.

The aim of the present study was to reassess with WGS, a cohort of *M. abscessus* isolated from CF patients previously genotyped with VNTR.

Materials and Methods

A total of 141 isolates of *M. abscessus* grown from sputum samples of 29 patients with CF had been identified at subspecies level by sequencing of *rpoB* gene.[21]

To perform VNTR analysis, we selected 13 *M. abscessus* loci, characterized by high allelic diversity, stability, and reproducibility, among the ones investigated in previous studies.[17-20] Loci with repeat size >25 and <50 bp and characterized by high discriminatory power, as determined basing on Hunter–Gaston index,[22] were privileged [Table 1]. PCR amplification and electrophoretic estimation of amplicon size were carried out as reported before.[20]

For WGS, we used the Illumina MiSeq sequencer, Nextera XT library preparation kits, and MiSeq reagent kits, following manufacturer's instructions (Illumina, USA). The reads were mapped to the reference genome of *M. abscessus* ATCC 19977 (GenBank ID: NC_010397.1) with the alignment program BWA,[23] and mappings were refined with the GATK[24] and SAMtools toolkits.[25] For variant detection in mapped reads were employed custom perl scripts with thresholds of a minimum coverage of four reads in both forward and reverse orientation, four reads calling the allele with at least a Phred score of 20 and 75% allele frequency. Single nucleotide polymorphism (SNP) positions with a reliable base call in at least 95% of the isolates were concatenated to a sequence alignment, excluding SNPs within a window of 12 bp from each other. From the aligned sequences of concatenated SNPs, a maximum parsimony tree was built on 1105001 SNP positions. The isolates fully sequenced were 95; each patient had at least one genome sequenced. For patients with multiple isolates, a proportion of them, including at least the first and the last one, was selected for WGS.

Results

Seven patients had a single isolate; the remaining 22 had multiple isolates, ranging from 2 to 19. Out of 22 patients with multiple isolates, 19 were persistently infected by a single subspecies: Masa in 13, Masb in 5, and Masm in 1. Three patients yielded isolates of two subspecies: Masm and Masa in two of them, Masb and Masa in the other.

VNTR analysis produced 29 different profiles, 15 patients had the same number of unique VNTR patterns, whereas the remaining five profiles were presented by more than one patient each [Table 2]. The variability of the number of repeats was significantly higher in Masa, with an average of 5.7 repeats/locus, in comparison with Masb (2.4) and Masm (1.6). Masb had steadily one repeat at locus Masb1; all the strains of Masm had one repeat at loci Mab1 and TR155 and two repeats at loci Mab11, Mab14, and Mab21.

Table 1: VNTR loci targeted in previous, and present, studies. In each row are reported the labels used by different authors for each single repeat

Choi *et al.*, 2011	Harris *et al.*, 2012	Wong *et al.*, 2012	Shin *et al.*, 2013	Present study
		TR2	Mab1	Mab1
		TR45	Mab4	Mab4
	2220		Mab7	Mab7
			Mab9	Mab9
VNTR11		TR116	Mab11	Mab11
	3163	TR131	Mab14	Mab14
	3416	TR150	Mab18	Mab18
		TR163	Mab21	Mab21
VNTR23			Mab23	Mab23
	4093	TR172	Mab24	Mab24
	4356		Mab28	Mab28
		TR200	Mab29	Mab29
		TR109		TR109
		TR155		TR155
	4038			
	3320	TR139		
	2177	TR86		
	3398			
		TR167		
		TR179		
		TR28		
		TR101		
		TR149		
		TR137		

Table 2: VNTR profile shared by more than one patient

VNTR profile[a]	*M. abscessus* subspecies	Patients (number of isolates)
5324433434233	*abscessus*	BF (3), BE (6), BO (1), CP (16), DE (1), FJ (4), GM (3)
4203232432231	*abscessus*	FI (4), TE (6)
4223331247434	*abscessus*	BS (29, PL (2)
1122221241231	*massiliense*[b]	BN (4), NO (1)*
1203221202321	*massiliense*	BS (2), CF (2)

[a]the digits indicate the number of repeats, the order of loci is the same as in Table 1 [b]identification on the basis of the WGS but contrasting with the *rpoB* sequence *strain transmitted between the two patients

In 18 out of 19 patients with multiple isolates belonging to the same subspecies, the VNTR profile remained unchanged over time suggesting persistent infection by a single strain; in the latter patient, a dominant strain, as indicated by five isolates with steady VNTR pattern, was occasionally overgrown by two isolates, each characterized by a unique profile. Interestingly, two patients (BN and NO) shared a VNTR profile presenting the signature of Masm despite the identification as Masa on the basis of the *rpoB* sequence.

The phylogenetic tree [Figure 1] showed three clearly distinct branches corresponding to the three *M. abscessus* subspecies; in it the strains of the patients above (BN and NO) belonged to the branch of Masm. The cluster corresponding to the subspecies Masa presented 35 closely related genomes [Figure 1, dotted frame] and 27 dispersed.

The same patient genomes differed for a number of SNPs ranging from 0 to 21 in 95% of patients; we considered,

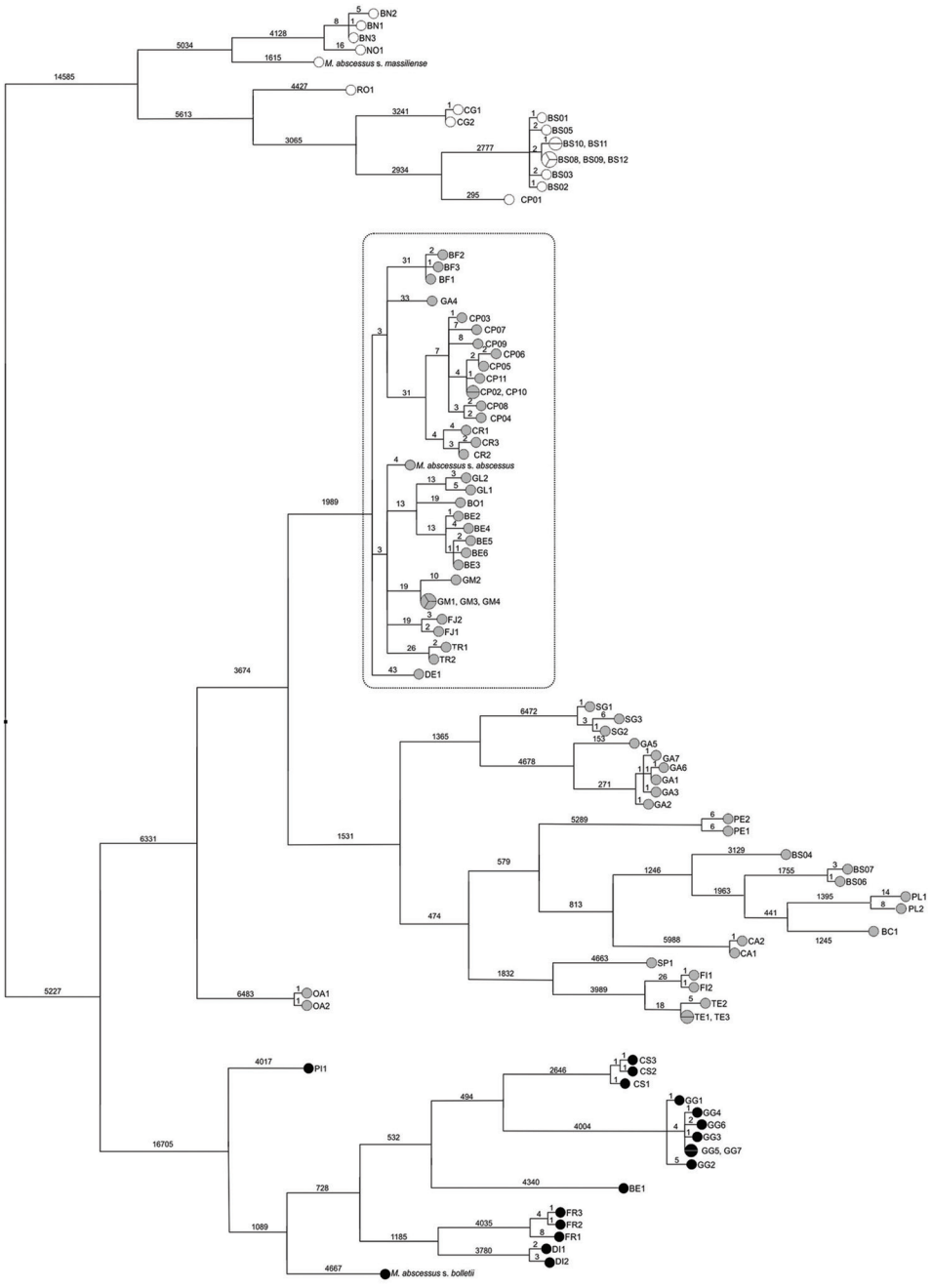

Figure 1: Phylogenetic tree including 95 clinical isolates (plus 3 reference strains) of *M. abscessus*. White circles indicate *M. abscessus* subsp. *massiliense*; gray circles indicate *M. abscessus* subsp. *abscessus*; black circles indicates *M. abscessus* subsp. *bolletii*; the dotted boxes highlight the dominating clone of *M. abscessus* subsp. *abscessus*. Numbers on branches indicate the number of distinct single nucleotide polymorphism positions between isolates. Same-patients isolates are indicated by unique two-letters identifiers followed by a number featuring the isolation order.

therefore, thirty SNPs a reasonable cutoff to distinguish isolates of the same strain from those of independent strains. On such basis, two patients (BN and NO) turned out infected by the same strain (difference 24–29 SNPs). WGS analysis showed furthermore that the six patients sharing the same VNTR profile [Table 2] were instead infected by independent, although closely related, strains. They differed in fact for 33–92 SNPs.

Discussion and Conclusions

As expected, in our comparison, the WGS revealed more discriminative than 13 loci VNTR. The subspecies Masa was the most affected by this deficiency of VNTR. In fact, a large number of Masa strains belonged to one dominating clone circulating worldwide.[5] Out of 11 patients infected by strains belonging to this clone, 7 had identical VNTR genotype [Table 2] and the others (GA, CR, GL, and TR) showed only one locus of difference. It is commonly accepted that VNTR patterns differing for the repeat number at one locus only can be regarded as identical,[18] and we have indeed observed one locus difference in one patient that we considered persistently infected by a single strain. Applying this rule, the number of patients infected by strains indistinguishable by our VNTR analysis, but demonstrated independent by WGS, would increase to 12, corresponding to 75% of the ones infected by Masa. Data excluding any epidemiological link among them further support WGS results. The only true case of transmission concerned a strain of Masm isolated from two patients pertaining to the same CF center and was correctly identified by both VNTR and WGS analysis.

In conclusion, we have observed that SNPs analysis has a higher discriminatory capacity compared to VNTR, in particular for Masa strains belonging to dominant circulating clusters. For other two *M. abscessus* subspecies, which however included here a limited number of isolates, no significant difference emerged between the two molecular approaches. Both methods revealed more accurate than *rpoB* gene sequencing in differentiating the strains at subspecies level.

Financial support and sponsorship

This study was supported by research grant FFC #27/2014 from Fondazione Ricerca Fibrosi Cistica.

Conflicts of interest

There are no conflicts of interest.

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