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Mycobacterium kumamotonense, another Member of the *Mycobacterium terrae* Complex Unusually Carrying Two Copies of the Ribosomal RNA Operon

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Abstract

The novel slow-grower *Mycobacterium kumamotonense* was previously misidentified as *Mycobacterium tuberculosis* complex using commercial probes. Similarly to other slow-growers that cross-react with the tubercle bacilli using commercial probes, *M. kumamotonense* is carrying two copies of the ribosomal RNA operon (*rrn*) per genome. Analysis of the corresponding *rrn* regions allowed the identification of sequences putatively involved in that cross-reactivity.

Keywords: *Mycobacterium kumamotonense*; Misidentification; ITS; *rrn* operons

Introduction

Different molecular probes, available to identify panels of mycobacterial species, have been developed and are widely applied in the diagnosis of the infections caused by these bacteria [1]. However, the misidentification could be found due to cross-reaction between probes and genomes of other species. Tortoli and co-workers described a frequent misidentification of commercial probes when less-frequently isolated species are present in the clinical sample. These authors found that some infrequent species were even not identified as members of the genus Mycobacterium by using one of the commercial probes tested [2].

The incorrect identification of an isolate as member of the Mycobacterium tuberculosis complex (MTBC) could carry main troubles in the diagnostic procedures, particularly when infrequently encountered species are involved as it could be the case if the aetiological agent was an emerging pathogen [2].

In a previous study, a clinical isolate, from a HIV positive patient diagnosed of lymphoid tuberculosis, was initially identified as member of MTBC by using AccuProbe (BioMérieux, Spain) and treated according to standard regimes. The un-complete recover of the patient prompted for a further characterization of the isolate by using standard microbiological procedures. These procedures allowed the identification of the isolate as *Mycobacterium kumamotonense* [3]. With the aim of clarify the previous result using AccuProbe, other two commercial probes were applied, however, one of them again misidentified the bacteria as MTBC. The three commercial probes applied have different genome regions as targets, all them belonging to the mycobacterial ribosomal RNA operon *rrn* [3].

In order to analyse these results more in deep, we focus on the study of the *rrn* operon including the corresponding internal transcribed spacer 1 sequences (ITS-1 corresponding to the 16S-23S ribosomal RNA intergenic region). We found that *M. kumamotonense* carried two copies of *rrn* per genome, which is unusual in slow grower mycobacteria. We were able to identify a sequence putatively involved in the cross-reactivity detected, within the ITS-1 region of several slow-growers.

Materials and Methods

Restriction fragment length polymorphism (RFLP) of the 16S rDNA was performed following previously described procedures [4].

The ITS-1 sequence (Internal Transcribed Spacer-1) corresponding to 16S-23S ribosomal RNA intergenic region, was analysed following the procedure described by Roth and co-workers [5]. The PCR amplified ITS-1 product was cloned into pGEM-T Easy vector (Promega Corporation, USA). Up to ten different colonies were selected for plasmid isolation and sequencing of the cloned fragments. Plasmids were sequenced with T7 and SP6 universal primers and sequences were analysed using 4Peaks software (v1.7.2, 4Peaks by A. Griekspoor and Tom Groothuis). ITS-1 sequences were aligned using the Mega software (v5.05).

Results and Discussion

According to the 16S rDNA sequence, the emerging pathogen *M. kumamotonense* was closely related to members of the *M. terrae* complex [6]. Interestingly, *M. terrae* carry two copies of the *rrn* operon per genome, which is unusual among members of this group [7]. This result prompted us to determine the number of *rrn* operons per genome of the *M. kumamotonense* under study. The RFLP pattern of the 16S rDNA gene showed two bands after BamHI digestion, thus indicating that, similarly to *M. celatum* and *M. terrae*, *M. kumamotonense* carried two copies of the ribosomal RNA operon per genome (Figure 1).

Previous publications showed misidentification of *M. terrae* and *M. celatum* as *M. tuberculosis* complex by using different commercial probes [8-11]. To have insights into that cross-hybridization, we performed the characterization of the two main targets of the

commercial probes found to cross-hybridize, namely 16S rDNA and ITS-1. Both targets are part of the ribosomal RNA operon (*rrn*).

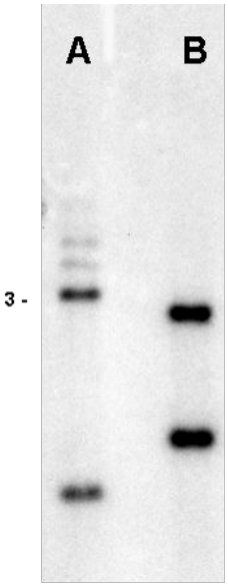

Figure 1: 16S rDNA RFLP using BamHI digested DNA from the following mycobacteria: A, rapidly grower *Mycobacterium fortuitum*; B, slow grower *M. kumamotonense*. The probe used targets a conserved region of the 16S rDNA gene, and hybridizes with a single fragment of that gene after digestion with BamHI. Fragment sizes (in kbp) are indicated on the left.

We have compared the 16S rDNA hypervariable region of members of the *M. terrae* complex and *M. celatum* [12]. In that region, *M. kumamotonense* showed 100% of similarity with members of the *M. terrae* complex, such as *M. terrae*, *M. senuense* and *M. algericum* and less than 94.3% similarity when compared to that gene of other mycobacteria, including *M. tuberculosis* and *M. celatum* (Table 1) [13,14]. We could not identify any sequence putatively involved in the cross-hybridization detected by using the commercial probe that targets 16S rRNA.

Higher variability was expected comparing sequences of the ITS-1 genomic region. After cloning and sequencing the ITS-1 regions of *M. kumamotonense*, two different ITS fragments of the same size (311 bp) were identified. The two ITS were named ITS-A and ITS-1B (Accession Numbers: FN597646 and FN597647 respectively). The sequences of these two fragments showed 92% of similarity to each other (Table 2). High level of similarity (99.6%) was also found comparing each other the two copies of ITS-1 described in the genome of *M. celatum* (Table 2). On the other hand, the two ITS sequences of *M. kumamotonense* showed a range of similarity between 67%-89% compared to other ITS sequences identified in mycobacteria, such as *M. tuberculosis*, *M. celatum*, and other members of the *M. terrae* complex (Table 2).

With the aim of identify the sequences that could explain the detected cross-hybridization, we analysed the sequence alignments of the complete ITS-1 region of *M. tuberculosis* and other mycobacteria, including the two sequences of *M. kumamotonense* (this work). Two putative regions of similarity were identified (21 and 25 nt length respectively) that could explain the cross-reaction described (Figure 2). These regions were 100% identical comparing *M. tuberculosis* H37Rv with *M. kumamotonense* (both, ITS-1A and ITS-1B), *M. celatum* (both, ITS-1A and ITS-1B), and M. terrae ITS-I, the three mycobacteria known that cross-hybridize with MTBC by using commercial probes (Figure 2).

The RFLP pattern found together to the two different copies of ITS identified in *M. kumamotonense*, showed the presence of two copies of the *rrn* operon in the genome of this slow grower mycobacteria (Figure 1 and Table 2).

		1	2	3	4	5	6	7
1	M. kumamotonense	100%						
2	M. aurupense	89.80%	100%					
3	M. terrae	100%	89.90%	100%				
4	M. senuense	100%	89.80%	100%	100%			
5	M. algericum	100%	89.80%	100%	100%	100%		
6	M. celatum	91%	85.50%	91%	91%	91%	100%	
7	M. tuberculosis H37Rv	94.40%	89.80%	94.40%	94.40%	94.40%	95.45%	100%

Table 1: Percentage of sequence similarity of the hypervariable region A of the 16S rRNA. Percentages of similarity between slow grower mycobacteria members of the *M. terrae* complex, including *M. kumamotonense*. Percentage of similarity with *M. tuberculosis* is also showed. Accession number of the sequences for slow grower mycobacteria used for comparison: *M. kumamotonense*, AB239925; *M. aurupense*, AB239926; *M. terrae*, X52925; *M. senuense*, JN571174; *M. algericum* NR117529.1; *M. celatum*, EFL08170; *M. tuberculosis* , X52917.

According to our data in *M. kumamotonense*, together to the previous data in *M. terrae* and *M. celatum* the presence of a supplementary copy of the *rrn* operon in slow grower mycobacteria could be related at some stage to the cross-reaction of these mycobacteria with probes developed to identify members of the MTBC. Out of the *M. terrae* complex and together to *M. celatum,* also the slow-grower mycobacteria *M. riyadhense* show cross-reaction with MTBC commercial probes [8-11,15]. Unfortunately, no data on the *rrn* content per genome is available of that mycobacterium thus far.

		1	2	3	4	5	6	7
1	ITS-1A M. kumamotonense	100%						
2	ITS-1B M. kumamotonense	92%	100%					
3	ITS M. aurupense	88.40%	77.40%	100%				
4	ITS M. terrae (Genotype I)	79%	71.30%	77.20%	100%			
5	ITS-1A M. celatum	77.90%	75.60%	72.70%	68.50%	100%		
6	ITS-1B M. celatum	76.50%	71.30%	73.10%	65.50%	99.60%	100%	
7	ITS M. tuberculosis H37Rv	67.80%	72.30%	69.50%	66.80%	69.40%	66.10%	100%

Table 2: Percentage of sequence similarity of the ITS-1 genomic region. Percentages of similarity between slow grower mycobacteria members of the M. terrae complex, including M. kumamotonense. Percentage of similarity with M. tuberculosis is also showed. The sequences of the two copies of M. celatum and M. kumamotonense are included (labelled as "A" and "B" respectively).Accession number of the sequences of slow grower mycobacteria used for comparison: M. kumamotonense, this work; M. aurupense, DQ523527; M. terrae (genotype I, 11), AJ314868; M. celatum, EF613281 (ITS-1A) and EF613282 (ITS1B); M. tuberculosis, NC000962.

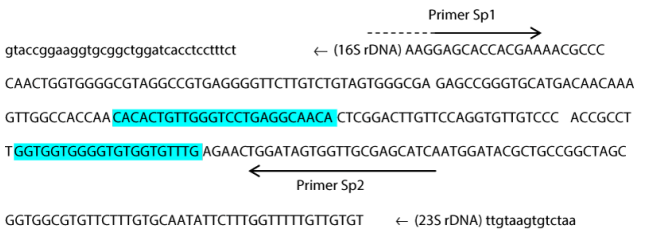

Figure 2: ITS-1 region of the M. tuberculosis H37Rv genome. The putative sequences that cross-react with M. tuberculosis are highlighted in cyan. The primers Sp1 and Sp2 are shown.

Rapid grower mycobacteria are by majority carrying two copies of the *rrn* operon per genome [16]. Contrary to slow growers, and with the exception of the rapid grower *M. holsaticum*, no cross-hybridization has been described at the moment between MTBC commercial probes and any other rapid grower mycobacteria [2]. This is surprising, taking into account the putative relationships of the number of *rrn* operons and cross-hybridization with MTBC, mainly considering the wide distribution of these bacteria and their frequent isolation from clinical samples.

The association of the second copy of the *rrn* operon in slow-growers with abnormal reaction to commercial probes was striking. More analyses are required to disclose the reason, if any, that links cross-hybridization with M. tuberculosis and number of copies of the *rrn* operon per genome in slow grower mycobacteria. Any suggested hypothesis is by now speculative.

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