

## Genomic Characterization of the Vaccinal Strain of *Mycobacterium avium* Subspecies *paratuberculosis* (MAP) 316F by MIRU-VNTR

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### ABSTRACT

**Background and Objective:** Paratuberculosis is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This study aimed to characterize the genome of the MAP 316F strain.

**Methods:** The MAP 316F strain was subjected to the PCR-F57 and PCR-IS900 experiments in order to ensure its identity as MAP. This was followed by application of the Thibault genotyping system consisting of eight loci including 292, x3, 25, 47, 3, 7, 10 and 32. Required genomic material for all experiments was prepared using the simple method of boiling. Gel electrophoresis findings related to the typing PCRs were backed by sequencing of amplification products.

**Results:** In PCR amplification, eight products with the size of 300, 298, 350, 217, 208, 203, 803 and 649 bp were detected at 292, X3, 25, 47, 3, 7, 10 and 32 loci, holding 3, 2, 3, 2, 2, 2 and 8 copies of TRs at these loci, respectively.

**Conclusion:** This genomic pattern is matched with that of the MAP 316F vaccine strain from the French Merial company and also the MAP K10 fully-sequenced strain.

**Keywords:** *Mycobacterium Avium* Subsp. *Paratuberculosis*, Genomics, Genotyping Techniques, Strain.

## INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes chronic enteritis in ruminants known as paratuberculosis. In Iran, the very early cases of this disease date back to the period of 1962-65 when paratuberculosis was diagnosed in pure-bred cattle of the Abadan oil company herd, imported from the UK (1). Many Iranian scientists, believed that imported animals are to be blamed for initial introduction and dissemination of paratuberculosis in this country. Considering the latest available epidemiological reports, paratuberculosis is currently scattered throughout Iranian cattle and sheep population in all or majority of the country's territory (2-5). MAP bacteria have a fastidious nature and their laboratory culture demands long-incubation time which in primary isolation of human and sheep cases might even exceed a year (6). Besides, successful bacterial culture of the pathogen is conditional to Iron supplementation (mycobactin J) of the culture medium. These difficulties have limited molecular epidemiological studies of MAP to fewer microbiology laboratories specialist in the field. Genomic studies have identified two principle classes (types) of MAP named sheep (S) and cattle (C) types. Molecular genomic methods have enabled the differentiation of these types (7, 8). In 2005, the result of full-genome sequencing of MAP K10 was released to the public domain (9). This virulent strain was initially isolated in 1970's from a dairy herd in Wisconsin, USA. As many as 4,829,781 bp and over 3,000 homolog genes with those from *Mycobacterium tuberculosis* were characterized in the MAP K10 genome (9).

Over the last two decades, the IS900-RFLP turned into the most broadly used method in genotyping of MAP. In 2007, Thibault pioneered in developing a variable number tandem repeat (VNTR) analyzing method based on 8 loci suitable for MAP genotyping. With receiving general acceptance from the scientific community, this method is turning to the standard protocol in epidemiological MAP Interspersed Repetitive Unit (MIRU) or VNTR

analysis. To date 139 genotypes have been deposited in the European VNTR database of *Mycobacterium avium* complex in France (available at: [mac-inmv.tours.inra.fr/](http://mac-inmv.tours.inra.fr/)) (10-14). MIRU-VNTR genotyping is a PCR-based system currently applied on numerous pathogenic bacteria (15-18) in which the copy number of specific genomic unit repeats, known as Tandem Repeats, in the genome of a given bacterium is determined. Depends on the copy numbers, size of the related genome segments differs among strains of bacterium. Technically, this is a straightforward and simple method that requires only a limited amount of genomic material with its simplified result format and amenable to digital processing and data-banking. These benefits have made MIRU-VNTR analysis superior compared to the older PFGE and RFLP genotyping protocols (19). In 1957, several laboratory MAP strains including 316F were moved from the Weybridge Institute, UK to Razi Vaccine & Serum Research Institute (RVSRI) by F. Entesar. Since then, MAP 316F has been used in production of paratuberculin (Johnin) and also paratuberculosis vaccine. More recently, this strain was resupplied to Razi by the Turkish collaborating center, Etlik. Since the properties of MAP 316F genome have not been yet studied, in this study the genomic characteristics of this strain have been analyzed by MIRU-VNTR typing.

## MATERIAL AND METHODS

Culture of bacterial strain and extraction of the genome: Two Falcon tubes containing Herrold's egg yolk medium supplemented with mycobactin J (20) were inoculated with Etlik MAP 316F sub-strain to supply enough bacterial mass. Incubation at 37°C lasted for 12 weeks until bacterial growth became visible. Bacterial genome was obtained by boiling as described previously (20). Genomic experiments: All genomic experiments were PCR-based and conducted in consecutive groups of identification and genotyping tests. For identification, the IS900 and F57 MAP bacteria specific markers were implemented. In PCR-IS900, Dohman's protocol (21) with few

modifications and in PCR-F57, Schonenbrucher protocol (22) using newly-designed primers were performed as the original protocol was not adaptable to conventional thermocyclers. For genotyping, the Thibault protocol was selected and applied to the 6 loci of 7, 3, 37, 25, X3, 292. For the two remaining loci of 10 and 32, new primer pairs were designed and applied as difficulties in amplification with Thibault's primers were experienced. A 2kb stretch of the MAP K10 genome flanking each of the loci was screened by Primer 3 version 4.0.0 (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>) to design primers of choice (23, 24) (Table 1). For PCR amplification, all loci were individually subjected to a quad-protocol amplification experiment with 24 separate reactions. These protocols were designed in a way that 6 different annealing temperatures (55, 56.7, 59.1, 60.4, 62.9 and 64.9 °C) were applied on PCR reactions containing 1 or 5 pmol of forward/reverse primers (Macrogen®, Korea) and 1 or 2.5 mM concentrations of MgCl<sub>2</sub>. PCR reactions, set at 15 µl volumes, contained a commercial master mix (Ampliquor®, Denmark). Double distilled PCR water was used as negative controls (Table 2). Gel electrophoresis of PCR products was performed using 1.5% multipurpose agarose gel, pre-stained with Redsafe®. Running of the loaded gels had continued for 2 hours at a 2 V/cm electrical field. Optimization of PCR reaction ingredients and cycles: Three different ingredient protocols were adapted for PCR-IS900, PCR-F57, the 6 MLVA-VNTR loci of 7, 3, 37, 25, X3, 292 and the 32 and 10 loci (Tables 1 & 2). Besides, two heating cycle protocols were developed; the first was applied to all

experiments and the second was used only the 32 and 10 loci. The mixtures from reactions classified in the first group were subjected to an initial denaturation at 95°C for 5 min followed by 35 amplification cycles of 30 s at 95°, 30 s at 61°C and 45 s at 72°C with a final extension treatment at 72°C lasting for 10 min. For MLVA-VNTR 32 and 10 loci, the amplification process was started with a heat treatment at 94°C continued for 5 min, followed by 35 consecutive cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 30 s complemented with a single final extension step of 72°C for 5 min. Precise sizing of PCR products and sequencing: extension treatment at 72°C lasting for 10 min. For MLVA-VNTR 32 and 10 loci, the amplification process was started with a heat treatment at 94°C continued for 5 min, followed by 35 consecutive cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 30 s complemented with a single final extension step of 72°C for 5 min. PCR products were sequenced in order to ensure the accuracy of gel electrophoresis results (Macrogen, South Korea). The raw data from sequencing were edited by Chromas Lite software version 2.1.1 (available on [www.technelysium.com.au](http://www.technelysium.com.au)) and the nucleotide structure of products was precisely determined through in situ alignment of the complimented forward and reverse sequence strings using Clustal X software version 2.1 (available on [www.clustal.org/clustal2/](http://www.clustal.org/clustal2/)). Detection of unit repeats was achieved by running the Tandem Repeat Finder software version 4.04 (available on <http://tandem.bu.edu/trf/trf.html>). No adjustments were made to the software default settings.

Table 1- primers used in this study

Locus/primer	Alias	Nucleotide sequence (5' → 3')	Start*	Stop*	Expected size (bp)*	Reference (s)
IS900f	-	TTC TTG AAG CGT GTT CGG GGC C	39815	39836	560	(21)
IS900r		GCG ATG ATC GCA GCG TCT TTG G	40353	40374		
F57f	-	ACC GAA TGT TGT TGT CAC CG	886774	886793	704	This study
F57r		GGA CAC CGA AGC ACA CTC TC	887458	887477		
MAP-292f	MIRU23 / MIRU2	CTT GAG CAG CTC GTA AAG CGT	3253590	3253610	300	
MAP-292r		GCT GTA TGA GGA AGT CTA TTC ATG G	3253865	3253889		
MAP-X3	VNTR1658 / MIRU3	AAC GAG AGG AAG AAC TAA GCC G	4441875	4441896	298	
MAP-X3		TTA CGG AGC AGG AAG GCC AGC GGG	4442047	4442070		
MAP- MIRU25f	-	GTC AAG GGA TCG GCG AGG	3665598	3665615	350	
MAP- MIRU25r		TGG ACT TGA GCA CGG TCA T	3665929	3665947		
MAP- MIRU47f	-	CGT TGC GAT TTC TGC GTA GC	4128605	4128624	217	
MAP- MIRU47r		GGT GAT GGT CGT GGT CAT CC	4128802	4128821		
MAP- VNTR3f	-	CAT ATC TGG CAT GGC TCC AG	131320	131339	208	
MAP- VNTR3r		ATC GTG TTG ACC CCA AAG AAA T	131506	131527		
MAP- VNTR7f	-	GAC AAC GAA ACC TAC CTC GTC	3711417	3711437	203	
MAP- VNTR7r		GTG AGC TGG CGG CCT AAC	3711602	3711619		
MAP- VNTR10f		CTC AAA GTC AGT GTG CCC GA	4279502	4279521	803	This study
MAP- VNTR10r		TCC TCT ACG CGG TCA TCT AC	4280285	4280304		
MAP- VNTR32f		GGA ACA CCA TGC CGA AGT TG	1125413	1125432	649	
MAP- VNTR32r		GCT AAG TTC CGG TTT GCC GA	1126042	1126061		

\*MAP K10 gene

Table 2- PCR amplification ingredients used in this study

PCR protocol	PCR master mix (µl)	Primer forward (5 pmol/µl)	Primer reverse (5 pmol/µl)	DNA template (µl)	DMSO (µl)	PCR water (µl)	Total volume (µl)
IS900, F57	7.5	0.5	0.5	4	0.6	1.9	15
292, X3, 25, 47, 3, 7	7.5	1	1	4	0.6	0.9	15
10, 32	7.5	0.25	0.25	5.25	0.6	1.15	15

## RESULTS

In PCR-IS900 and PCR-F57 amplification of MAP 316F, two fragments as large as 560 bp and 704 bp were produced respectively, to confirm its identity. In MIRU-VNTR typing of MAP 316F, gel electrophoresis findings, supported with sequencing, detected 8 fragments at PCR-VNTR292 (300 bp), X3 (298 bp), MIRU-VNTR 25 (350 bp), MIRU-VNTR 47 (216 bp), MIRU-VNTR 3 (208 bp), MIRU-VNTR 7 (203 bp), MIRU-VNTR 10 (803 bp) and MIRU-VNTR 32 (649 bp). The outcome of data processing from running Tandem Repeat Finder on these loci detected 3, 2, 3, 3, 2, 2, 2 and 8 copies of the unit repeat at VNTR292, X3, MIRU-VNTR 25, MIRU-VNTR 47, MIRU-VNTR 3, MIRU-VNTR 7, MIRU-VNTR 10 and MIRU-VNTR 32 loci, respectively. In a comparative in situ analysis of genomic data from MAP 316F strain and those matching segments from the MAP K10 genome, no differences either in unit repeat numbers or single nucleotide polymorphism (SNP) was identified. Investigating the MAP MIRU-VNTR genotyping databank in France (<http://mac-inmv.tours.inra.fr/>) revealed that the MAP 316F strain genotype, INMV2, was already registered with the database.

## DISCUSSION

Accurate identification and characterization of strains/isolates is a principle requirement in biological research and production settings. In 1920, at the Cornell University in the United States, a fast-growing mycobactin J - independent laboratory isolate of *Mycobacterium avium* complex came to the attention. For almost half a century, this strain, known as MAP 18, was mistaken by the scientific community for MAP until 1968 when it was officially withdrawn from American Type Culture Collection (25). This historic fault indicates the importance of specific genetic markers in accurate identification of microorganisms. Despite the specificity of IS900 due to its high similarity with those named as IS900-like, characterization of MAP-suspected bacteria, solely based on IS900 is prone to error (26, 27). Given the many years of archiving both

MAP 316F and *Mycobacterium avium* subspecies *avium* D4 strains at Razi and efforts to address the concerning possible cross-contamination of these two, in this study two MAP specific markers of IS900 and F57 were simultaneously employed. Up to the present, some of the PCR-IS900 and almost all the PCR-F57 based available strategies have been adapted to be used in real-time thermocyclers. These are typically expensive equipments which are not available in many veterinary microbiology laboratories. Moreover, genomic fragments of interest in real-time PCR are normally smaller than 100 bp in length, rendering them hard to be characterized by conventional gel electrophoresis. In this study therefore, a new pair of primers was designed to amplify a larger fragment (350 bp) carrying the F57 element. Besides, by developing a single protocol both PCR-F57 and PCR-IS900 could be performed in one PCR run. At least two sub-strains of MAP 316F are currently available, the British Weybridge and the French Merial sub-strains. In Thibault's study in 2007, both sub-strains were subjected to the MIRU-VNTR assessment and in consequence two different types were identified. The British sub-strain represented INMV17 (31332228) and the French sub-strain held INMV2 (32332228). While the Weybridge sub-strain genome carries a single copy of unit repeat at its X3 locus, genome of its Merial counterpart holds 2 copies of the unit repeat (6, 14). As it is represented here, the Razi sub-strain of MAP 136F, supplied by the Turkish Etlik Institute, similar to the French sub-strain, holds two copies of the unit repeat at its X3 locus. This similarity might be explained by a potential French supplier of the Turkish sub-strain or a mutual ancestor for both French and Turkish sub-strains. Since there is no data released on genotype of the Turkish sub-strain, we are unable to assess either of these scenarios.

## CONCLUSION

F57 and IS900 are examples of genetic markers useful in laboratory identification of MAP isolates where long incubation periods and/or culture requirements hinder isolation and mass culture of bacterium. The Razi sub-strain

of MAP 316F is genetically different from its Weybridge ancestral sub-strain at locus X3. Since the Turkish Etlik institute is the supplier of the current strain at Razi, we assume the detected genomic changes might have materialized before its arrival to Razi.

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#### CONFLICT OF INTEREST

There are no conflicts of interest.

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