Short Paper

First report of MIRU-VNTR genotyping of *Mycobacterium avium* subsp. *paratuberculosis* isolates from Egypt

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Summary

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, an economically important disease in ruminants worldwide. It was first isolated in Egypt in 2005. Since then, the pathogen has been detected in different Egyptian provinces. In order to trace the source of infection, genotyping using simple methods of high discriminatory power such as mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) were carried out in different countries. Until now there is no published information about MIRU-VNTR genotyping of MAP isolates in Egypt. To address that point, 100 faecal samples were collected and cultivated from 3 different suspected dairy farms. Fourteen isolates belonging to one farm were identified as MAP and subjected to genotyping using 8 different MIRU-VNTR loci PCRs. Two different genotypes were recognized based on size polymorphism observed in one locus (VNTR-7) that was confirmed by sequencing. Our work provides a preliminary basis of constructing a MIRU-VNTR genotyping database of MAP in Egypt.

Key words: Egypt, INMV, MIRU-VNTR, *paratuberculosis*

Introduction

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of Johne's disease, one of the most economically important diseases of ruminants worldwide (Losinger, 2005). It is also suspected to be associated with Crohn's disease in humans (Liverani *et al*., 2014). The pathogen was first isolated in Egypt in 2005 (Salem *et al*., 2005). Since then, our group, and others as well, had investigated the pathogen in different provinces (Fawzy *et al*., 2013; Abdellrazeq *et al*., 2014; Amin *et al*., 2015); however little is known about the genomic diversity of such organism at the national level.

Genotyping is a very important tool of epidemiology that helps to trace back the source of infection in case of outbreaks and surveillance programs (Sohal *et al*., 2009). One of the simple PCR-based methods that has a high resolution power is mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR). MIRU-VNTR are DNA locations distributed in the genome of mycobacteria, where at each location a certain nucleotide sequence is present in more than one copy arranged beside each other in a head to tail manner. The copy number of each MIRU-VNTR differs between epidemiologically unrelated strains due to mutations; therefore amplification of each location would give PCR products of different sizes upon gel electrophoresis (Biet *et al*., 2012). The aim of our work is to isolate MAP from suspected animals in different Egyptian provinces and to characterize the isolates using MIRU-VNTR genotyping. MAP was isolated from animals belonging to one farm and upon genotyping, two different strains were characterized.

Materials and Methods

Collection of samples

Faecal samples were collected from 100 cattle (Holstein-Friesian breed) aged between 2.5 and 4 years belonging to 3 different dairy farms in three different Egyptian provinces (Fayoum n=30, Giza n=27, and Ismaeleya n=43). The involved farms had a history of chronic intermittent diarrhea. Samples were collected randomly during the routine pregnancy testing by rectal palpation, shipped to the laboratory and either processed directly or stored in -20°C until processing.

MAP isolation

Isolation of MAP was performed according to Whipple *et al*. (1991) with slight modifications. Three g of the faecal samples were suspended in 30 ml freshly prepared 0.75% HPC (Sigma, Germany) in a 50 ml plastic tube that was shaken at 200 rpm for 30 min then kept for 1 min at room temperature. The supernatant was transferred into new serial plastic tube without disturbing the faecal sediment. The sample was kept for 18 h at room temperature in the dark, and centrifuged at 2.500 × g for 15 min. The supernatant was descanted and the

pellet was re-suspended in 1 ml HPC, then 200 µL was inoculated onto 3 tubes of HEYM containing mycobactin J and 1 tube of HEYM with no mycobactin, incubated at 37°C and checked for growth and/or contamination every week for up to 16 weeks. All isolates were subjected to confirmatory F*57* and IS*900*-based PCRs in order to confirm the identity of the isolates (Vansnick *et al*., 2004).

MIRU-VNTR genotyping

MIRU-VNTR PCRs targeting eight different loci were carried out. The primers and PCR conditions used were those described by Thibault *et al*. (2007) (VNTR 25, VNTR 47, VNTR 3, VNTR 7, VNTR 10, VNTR 32) and Bull *et al*. (2003) (MIRU 2, MIRU 3). Briefly, 20 µL final reaction volume contained 10 µL of Hotstar Taq MasterMix (Qiagen, Germany), 1 µL of each forward and reverse primer (10 pmol/µL), 6 µL DNase free PCR grade water, and 20 ng of the extracted DNA. The amplifications were carried out using a Biometra thermocycler (T3000, Germany). PCR products were subjected to gel electrophoresis (2% agarose) at 100 V for 1 h and according to the band size, the number of repeats at each locus was calculated. One representative of each allele was sent to Seqlab-Microsynth laboratories (Göttingen, Germany) for sequencing to check for repeat numbers. The sequences were aligned with T-Coffee software (http://tcoffee.crg.cat/apps/tcoffee/do:regular).

Results

Fourteen of the inoculated samples (only from Ismaeleya province) showed colonies after 6-8 weeks of incubation only on HEYM tubes with mycobactin J. The morphology of the colonies was identical to MAP isolates with pinpoint colorless appearance at first that later enlarged and became creamy opaquein color. All colonies were confirmed as MAP by the aforementioned confirmatory PCRs.

In MIRU-VNTR PCRs, all isolates revealed the same amplicon sizes for all loci investigated (Fig. 1) except for VNTR 7 locus where two different variants were observed (Fig. 2) that were confirmed by sequence analysis (Fig. 3). According to INMV nomenclature described by Thibault *et al*. (2007) that was based on the numerical expression of the repeat numbers at 8 different MIRU-VNTR loci, MAP isolates used in this study belong to two genotypes (INMV 1 and INMV 19) (Table 1).

Discussion

We report here for the first time data about MIRU-VNTR genotyping of *paratuberculosis* in Egypt, where 14 MAP isolates from different animals within one farm in Ismaeleya province had two different patterns. Our results confirm the high resolution of MIRU-VNTR genotyping tool as documented before (Biet *et al*., 2012) and refer to the presence of at least two different strains cycling in that farm (INMV 1 and INMV 19) (Table 1), a

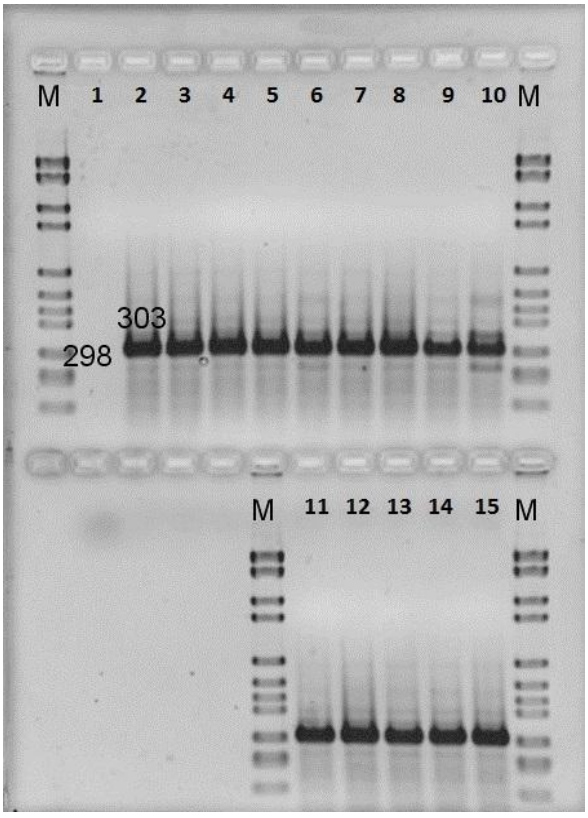

Fig. 1: VNTR 10 locus PCR showing no polymorphism. M: Marker. Lane 1: No template control; all isolates (Lanes 2-15) show the same 303 bp-sized band (two repeats)

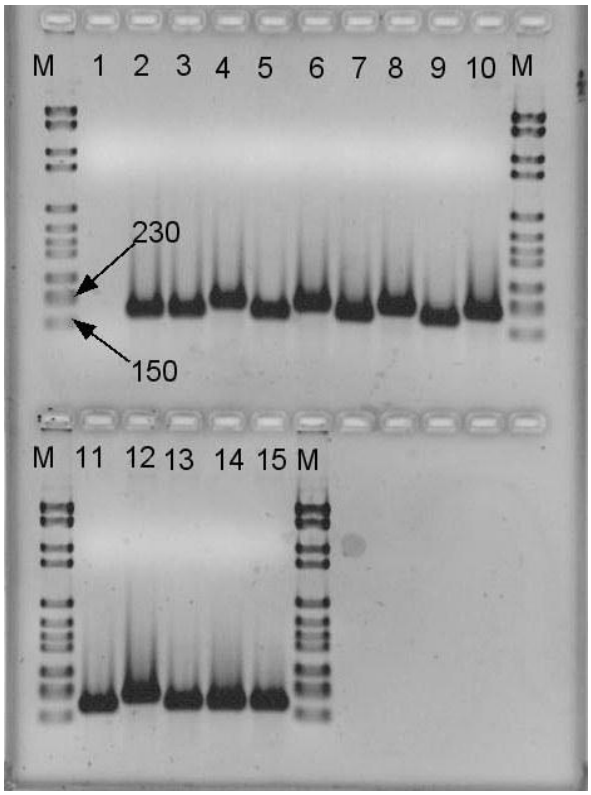

Fig. 2: VNTR 7 locus PCR showing polymorphism. M: Marker. Lane 1: No template control; Lanes (2, 3, 5, 7, 9, 11, 13, 14, 15) show 181 bp band (one repeat); Lanes (4, 6, 8, 10, 12) show 203 bp band (two repeats)

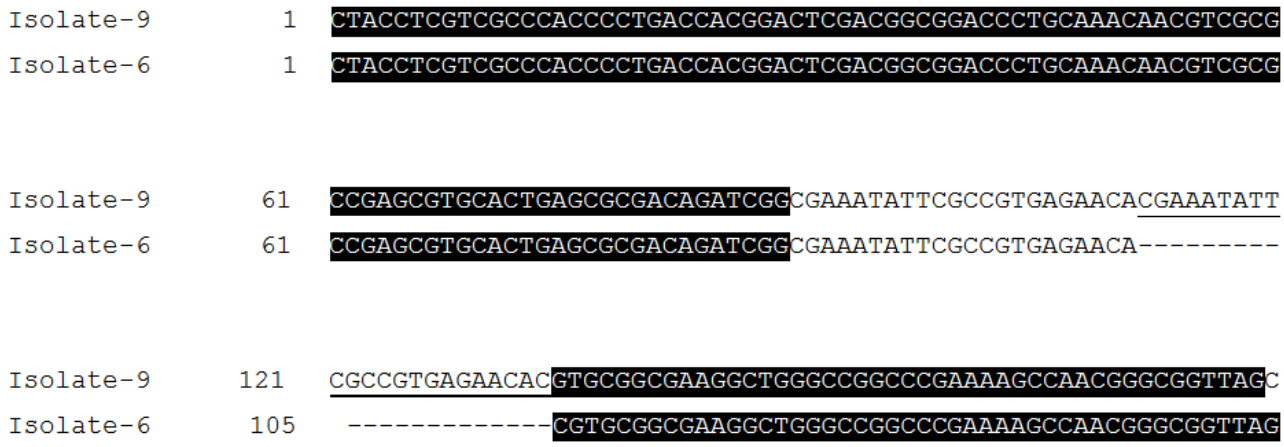

Fig. 3: Multiple sequence alignment of VNTR 7 partial sequences of two MAP isolates showing size polymorphism upon gel electrophoresis. The 22 bp tandem repeat sequence described by Thibault *et al*. (2007) is represented as black letters with no black shadow. Isolate-9 has two copies with the second copy being underlined and isolate-6 has only one copy

Table 1: Number of tandem repeats at MIRU-VNTR loci in the investigated MAP isolates

Number of isolates	Province	Number of MIRU-VNTR tandem repeats								INMV[a] nomenclature
		MIRU 2	MIRU 3	25	47	3	7	10	32	
9	Ismaeleya	4	2	3	3	2	1	2	8	INMV 19
5	Ismaeleya	4	2	3	3	2	2	2	8	INMV 1

[a] INMV nomenclature is a numerical expression of the number of tandem repeats of 8 different MIRU-VNTR loci (11)

situation that was reported before for *paratuberculosis* in the Netherlands (Hulzen *et al*., 2011), Spain (Castellanos *et al*., 2010) and Austria (Gerritsmann *et al*., 2014). This phenomenon could be due to two different infection sources or microevolution of the pathogen within the farm during the long incubation period of such a chronic disease (Sohal *et al*., 2014). However, by reviewing the trading history of the farm, the cattle were purchased from different sources which could favor separate introduction events as the cause of this phenomenon.

Johne's disease represents a threat to the ruminant industry worldwide. In African countries, the control of the disease is partially hindered by the lack of studies about the prevalence of the disease and the genomic diversity of the pathogen. However, some studies have reported the disease in some African countries (Michel *et al*., 2000; Okuni *et al*., 2013). Okuni (2013) presented a detailed review about the published data concerning *paratuberculosis* in Africa.

In Egypt, MAP was mainly isolated from exotic cattle breeds (Holstein-Friesian) as they are usually reared in dairy farms due to their high milk yield, however, it was also isolated from native cattle and buffalo breeds (Salem *et al*., 2005; Abdellrazeq *et al*., 2014; Amin *et al*., 2015). In a serological based study, MAP seropositivity was evident for native sheep and goat breeds, but the pathogen was never successfully isolated from small ruminants in Egypt (Fawzy *et al*., 2013). To the best of our knowledge this is the first attempt at genotyping MAP isolates originating from Egypt using MIRU-VNTR. Although our work is limited only to isolates obtained from one farm, it could provide a preliminary basis of constructing a MIRU-VNTR genotyping database of the pathogen in Egypt.

Conflict of interest

All authors declare that they have no conflict of interest.

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