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A first insight into the application of high discriminatory MIRU-VNTR typing using QIAxcel technology for genotyping *Mycobacterium bovis* isolated from the Delta area in Egypt

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ABSTRACT

Mycobacterium bovis is a notorious infectious agent leading to serious economic losses for cattle farms worldwide. Analysis of the widely spreading genotypes is vital for tracing infections, understanding transmission dynamics, and controlling the cluster growth. This study aimed to evaluate the discrimination ability of 15 mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) loci and to assess the extremely efficient loci subset for molecular epidemiological investigations of *M.bovis* from farms in the Delta area of Egypt. The discriminating ability of MIRU-VNTR genotyping using 15 loci {2 exact tandem repeat (ETR) loci, 6 MIRU loci, 4 Mtub loci, and 3 Queen's University of Belfast (QUB) group loci)} were evaluated on 61 *M.bovis* isolates from cattle (Holstein Frisian) and buffaloes. The results indicate that there are 48 genotypes: 3 unique genotypes and 45 genotypes with shared similarities. Using the MIRU-VNTRplus database, *M.bovis* ID 7540/01 and ID 5346/02 were the nearest lineages to both groups. Six loci (MIRU10, QUB11b, QUB26, ETRA, Mtub30, and Mtub39) were highly discriminating, seven other loci (Mtub21, MIRU26, QUB4156, MIRU04 (ETRD), MIRU16, MIRU 40, and ETRC) gave moderate discriminatory power, and the last two loci (Mtub04 and MIRU31) were poorly discriminative. MIRU-VNTR typing generally proved efficacy and high discriminatory power, with a collective allele's diversification of 0.9641. Both the six highly discriminating (DI = 0.9492) and the seven moderately discriminating loci (DI = 0.9269) evidenced to be suitable for *M.bovis* first-step initial genotyping from cattle herds in Egypt. MIRU-VNTR is rapid and effective in the genotyping of *M.bovis* from cattle and buffaloes in Egypt.

1. Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTC), leads to tuberculosis in bovines (BTB), a broad range of livestock and human infections, and great economic losses (Cezar et al., 2016; Franco et al., 2017). The genotypic analysis of MTC members is considered an essential epidemiological tool to study the spreading and control strategies against these members (Yang et al., 2015; Teeter et al., 2016). The mycobacterial VNTR loci were named variable number of tandem repeats (Jagielski et al., 2014). Mycobacterium genotyping is based on mycobacterial interspersed repetitive (VNTR-MIRUs), tandem repeat (ETR), Mtub and QUB which were respectively described by Supply et al. (2001), Hangombe et al. (2012), and Jagielski et al. (2014).

The introduction of molecular genotyping methods for *Mycobacterium* improved the knowledge of TB transmission dynamics, and bacterial evolution (Niemann and Supply, 2014). The MIRU-VNTR typing has the highest efficiency and reproducibility because the results expressed in a numerical form and can be easily compared between various laboratories (Jagielski et al., 2014). To obtain a firm genotypic resolution, there have been many attempts to investigate various VTNR loci the Queen's University Belfast (QUB), and Mtub groups (Supply et al., 2006). Various combinations of MIRU-VNTR loci have been reported to efficiently differentiate mycobacterial strains (Carvalho et al., 2016). Globally, the routine MTBC genotyping using MIRU-VNTR typing relying on a combined group of 24 loci has become a universal standard (Supply et al., 2006).

The QIAxcel technology offered an alternative to electrophoresis tools based on agar gels for separating and sizing PCR products (Matsumoto et al., 2013). To obtain reliable genotypic results, the Mtb genotyping techniques included the application of MIRU-VNTR genotyping, direct repeat locus-based spacer oligonucleotide typing (spoligotyping), single-nucleotide polymorphisms (SNPs), and whole-genome sequencing (WGS). The gained data from these techniques uncovered

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the MTBC populations, the internal natural variations, and the host pathogen relationship (Coll et al., 2014; Teeter et al., 2016).

Due to the lacked data about the most prevalent *M. bovis* genotypes in Egypt we studied the distribution of variable numbers of tandem repeats in isolates from cattle and buffaloes in the Delta area of Egypt.

2. Materials and methods

2.1. Animal testing, bacterial isolation, and DNA extraction

2.1.1. Tuberculin test

A total of 2100 farm animals {Holstein Friesian (HF) and buffaloes} from different Egyptian Governorates during the period of 2011–2016 were tested by single intradermal comparative cervical tuberculin (SICCT) (Bezos et al., 2014). The samples from organ tissues and lymph nodes were collected from different animals. The study design was accepted by the Faculty Committee for Animal Care and Use, relevant to the Faculty of Veterinary Medicine, University of Sadat City.

2.1.2. Sampling and cultivation

The prepared samples were decontaminated by the modified Petroff's method. The cultivation, isolation and identification steps were performed according to Elsayed et al. (2016).

2.1.3. The DNA extraction and genotyping

The DNA extraction was performed using a combination between the enzymatic, physical, and chemical processes, as described by Romano et al. (2005).

2.2. Molecular detection of the MTC members

2.2.1. Simplex real-time PCR using the atpE primer and probe

The mycobacterial quantification by real-time PCR targeting *atp*E gene was performed as described by Radomski et al. (2013). The primers and probes were synthesized and supplied by Applied Biosystems, Foster City, Calif., The purified DNA of *M.bovis* BCG (ATCC19210) and *M.tuberculosis* H37RV were used as positive control isolates, and the concentrations were {100 000, 1000, 100, 10, 1, 0.1 and 0.01 genome equivalents (ge)} that were confirmed by Quant-iT™ RNA Assay Kit with the Qubit® fluorometer.

2.2.2. Multiplex conventional PCR

The multiplex and simplex conventional PCR techniques targeting the regions of difference (RDs) were performed according to Warren et al. (2006).

2.3. MIRU-VNTR typing

2.3.1. The primers used for MIRU-VNTR

The primers used for MIRU-VNTR were similar to those applied for *M.tuberculosis* H37Rv. Eight primers used in this study were similar to some JATA primers (Wada and Maeda, 2013): J1:424 (Mtub04), J2:960 (MIRU10), J3:1955 (Mtub21), J5:2163b (QUB11b), J7:2996 (MIRU26), J9:3192 (MIRU31), J11:4052 (QUB 26), and J12:4156 (V4156) QUB4156. These primers were synthesized by (Takara Holdings, Japan).

2.3.2. Other primers set

Seven primers used in previous studies by Supply et al. (2006) and Wada and Maeda (2013) were included in the present study: 580 (MIRU 04), 1644 (MIRU 16), 802 (MIRU 40), 2156 (ETRA), 577 (ETRC), 2401 (Mtub30), and 3690 (Mtub39), these primers were synthesized by (Takara Holdings, Japan).

2.4. The calculations of allelic diversity (h) and the discriminatory index (DI) of MIRU-VNTR typing

The equation h = n (1 − Σxi2)/(n − 1) was used to calculate the allelic diversity (h) of different applied MIRU-VNTR loci, as n represents the total number of isolates and xi is the ith allele frequency at specific locus (Kim et al., 2010). The discriminatory index (DI) was calculated according to the following formula of Hunter and Gaston (1988):

$$DI = 1\text{-}1/N(N\text{-}1)\sum_{j=1}^{s} N_j(N_j\text{-}1),$$

which proves the probability that the MIRU-VNTR typing will assign two randomly sampled unrelated strains within the microbial population into different groups. The online tools at http://www.MIRU-VNTRplus.org were used to complete the discriminatory index (DI) for all tested VNTR loci and the combinations used.

3. Results and discussion

In this study, it was found that 81/2100 (3.8%) of the tested animals were tuberculin positive (Table 1), which correlated with the previous records of Cosivi et al. (1998) who proved that the rate of tuberculin positive cattle and buffaloes ranged from 2 to 9%. The result obtained surpassed the recent records during the year 2015 and 2016 (Elsayed et al., 2016). This result could be interpreted by the uncontrolled animal movements and trading between different Governorates during this period.

Table 1
Tuberculin testing, isolation, and confirmation of *M.bovis* from different animal species in various Egyptian localities.

Locality	Species	Tested animals	Date of testing	Tuberculin test		Isolation on LJ with sodium pyruvate	Molecular confirmation	
				+VE	%		Real-time PCR using *atp*E	Multiplex and simplex conventional PCR targeting different (RDs)
Menoufia	Cattle	400	January 2016	7	1.75	7	61	61
		130	June 2011	4	3.07	4		
	Buffalo	500	April 2013	27	5.4	7		
Sharkia	Cattle	180	May 2014	9	5	9		
Gharbia	Cattle	100	March 2011	4	4	4		
		80	June 2011	2	2.5	2		
		120	May 2015	5	4.16	5		
Dakahlia	Cattle	60	May 2015	2	3.33	2		
	Buffalo	30	May 2015	1	3.33	1		
El-Buhaira	Cattle	450	August 2012	18	4	18		
Cairo	Cattle	50	November 2012	2	4	2		
Total		2100		81	3.8	61/81 (75.3%)	61/81 (75.3%)	61/81 (75.3%)

The PCR amplification of the genomic RDs was essential for differentiation of MTBC members. Simplex PCR targeting (RD4 and RD9) and simplex real-time PCR targeting *atp*E gave clearer results and confirmed that 61/81 (75.3%) isolates were *M.bovis* (Warren et al., 2006; Radomski et al., 2013) (Table 1).

The 15 MIRU-VNTR loci discriminatory capacity was investigated. With specific focus on locus Mtub30 (Supplementary Material Table 1), it gave the greatest individual DI (0.7393), then both ETRA and MIRU10 which correlate with a previous study on ETRA (Yang et al., 2015). The MIRU-VNTR loci used in this study exhibited higher h than that in an earlier report from the People's Republic of China (Yang et al., 2015). The V4156 showed nearly similar h to that obtained in a previous study by Supply et al. (2006). This study is the first to utilize 15 VNTR loci for *M.bovis* genotyping in Egypt. The used methodology proved a comparatively higher discriminatory index (DI) and lower rates of clustering than results gained by Yang et al. (2015). Comparing the implementation of the 15 loci with the 12 loci, it was clear that the 15 loci subset used conferred greater genotyping resolution (Yang et al., 2015).

Moreover, the gained discriminating capacity of the 15 loci subset exhibited variation with a high DI. The utilized MIRU10, QUB11b, QUB26, ETRA, Mtub30, and Mtub39 loci combination provided the greatest resolution (DI = 0.9492) (Supplementary Material Table 2), while the combined moderately discriminatory MIRUs {Mtub21, MIRU26, QUB4156, MIRU04 (ETRD), MIRU16, MIRU 40, and ETRC} had lower resolution (DI = 0.9269). On the other hand, the Mtub04 and MIRU31 (ETRE) provided the lowest resolution (DI = 0.5586). Hence, the discriminating capacity of the 15 loci was greatly enhanced by making modern combinations of these loci. It was clear that application of 15 VNTR loci-based typing technique is a highly discriminatory method for the initial genotyping of *M.bovis* isolated from cattle and water buffalo from Egypt, replacing the original 12 MIRU loci.

The discriminatory index of 0.95 is the lower theoretical limit of a molecular marker for epidemiological inferences to be acceptable in a molecular epidemiological study. As such with 0.9641, the current MIRU-VNTR loci choice is just above this threshold.

Furthermore, for in-depth studies, it is recommended that MIRU10, QUB11b, QUB26, ETRA, Mtub30, and Mtub39 loci combination be used as a first-line set of loci for gaining rapid data and for helping first-line molecular epidemiology by anticipating the genotyping results using the full set of MIRU-VNTR loci. Relying on the non-zero h results of the 15 MIRU-VNTR loci, a total of 48 genotypes were obtained and classified into 30 diverse types (DI = 0.9641), 3 unique types, and 27 clustered types. The clusters harbored 1 (n = 1) to 2 (n = 4) analogous isolates (Supplementary Material Table 3) and Fig. 1. Focusing on the obtained genotypes, GT29 and GT32 exhibited the highest rate of distribution, (5/48) 10.41%. G29 was widely distributed amongst HF cattle farms in Cairo, Gharbia, and Minufyia, while GT32 was found widely in Al buhaira Governorate HF farms. Moreover, GT2, G11, and GT33 had a similar rate, 2/48 (4.16%), of the distribution on HF farms in Gharbia and Al buhaira Governorates. In contrast, the other genotypes, G1, G3-G10, G12-G25, G27, 28, 30, 31, and G34-G43, constituted the lowest rate, 1/48 (2.08%), of the distribution from HF cattle. Additionally, G26 and G44-G48 exhibited the lowest rate, 1/48 (2.08%), of distribution from native buffalo within Dakahlia and Menoufia Governorates. Based on the genotype similarities, *M.bovis* ID: 7540/01 was the nearest lineage to 3 of our genotypes, while the other 45 genotypes were nearest to the *M.bovis* ID: 5346/02 lineage by the 15 MIRU-VNTR loci and the MIRU-VNTRplus database.

Being the first report of its kind, this study presents results that will be beneficial for disease control authorities and health departments to assess easily linked disease clusters from an epidemiological point of view. These data will help in understanding the transmission dynamics and cluster growth of *M.bovis* and will be valuable in identifying risk factors, and tracing infections.

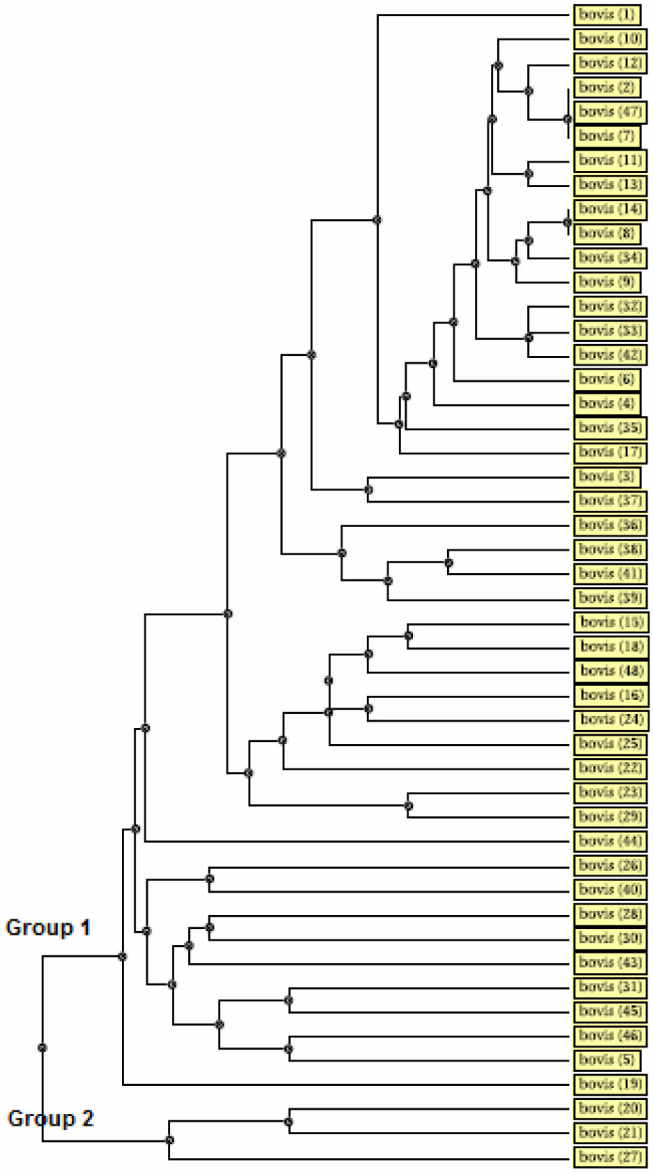

Fig. 1. Dendrogram of MIRU-VNTR typing of the obtained 48 *M.bovis* genotypes. Two groups of *M.bovis* isolates were gained, group 1 contained 45 genotypes with shared similarities and group 2 contained 3 unique genotypes, the clusters harbored 1 (n = 1) to 2 (n = 4) analogous isolates. *M.bovis* ID: 7540/01 was the nearest lineage to 3 of our genotypes, while the other 45 genotypes were nearest to the *M.bovis* ID: 5346/02 lineage by the 15 MIRU-VNTR loci and the MIRU-VNTRplus database using the online tools at http://www.MIRU-VNTRplus.org

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.04.004.

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