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Mycobacterial Interspersed Repetitive Unit-variable Number Tandem Repeat (MIRU-VNTR) Typing Lacks Discriminatory Power in the Genetic Analysis of Bovine Tuberculosis in Egypt

Hebatallah Ahmed Mahgoub[1,*], Mohamed F. Salama[2], Mohamed El-Adl[2], Ahmed Ammar[3], Ali Amer[4], Samar Atwa[5], Youssef Alseady[6], Walaa Awadin[1], Emad Mokhtar[4]

[1]Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Egypt
[2]Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Egypt
[3]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Zagazig University, Egypt
[4]Tuberculosis Department, Animal Health Research Centre, Egypt
[5]Department of Internal Medicine, Infectious and Fish Diseases, Faculty of Veterinary Medicine, Mansoura University, Egypt
[6]Physiology Department, Faculty of Veterinary Medicine, Mansoura University, Egypt
*Corresponding author: hebamahgoub@mans.edu.eg, heba.a.mahgoub@gmail.com

Abstract Tuberculosis (TB) is one of the most important infectious zoonotic diseases of vertebrates worldwide. TB in animals is primarily known from cases in cattle and other bovids for which the disease is generally referred to as bovine TB. The major causative agent of bovine TB is *Mycobacterium bovis* (*M. bovis*), a member of the *Mycobacterium tuberculosis* complex. Animal TB is a disease of high economic relevance within the context of livestock farming as it directly affects animal productivity and also influences international trade of animal products. In this study, we aimed at mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis of cases of TB infection in local cattle in Egypt. Therefore, various samples (milk and blood samples) were collected from cattle farms (in Damietta Province) that were positive for tuberculin test. Mycobacterial isolation was tested on milk samples, but it showed negative result. DNA was extracted from blood samples. Five mycobacterial genes (*IS6110*, *katG*, *gyrA*, *oxyR*, *pncA*) were used for further confirmation of field TB infection. All blood samples were positive for Mycobacterial-specific genes. Twelve MIRU-VNTR loci were used to test their discriminatory power in the genetic analysis of TB, but such MIRU-VNTR loci typing failed to show any discriminatory power for the genetic analysis of bovine TB cases.

Keywords: *bovine tuberculosis, MIRU-VNTR typing, tuberculin test*

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1. Introduction

Tuberculosis (TB) in animals is primarily known from cases in cattle and other bovids for which the disease is generally referred to as bovine TB [1,2]. The major causative agent of bovine TB is *Mycobacterium bovis* (*M. bovis*), a member of the *Mycobacterium tuberculosis* complex. Animal TB is a disease of high economic relevance within the context of livestock farming as it directly affects animal productivity and also influences international trade of animal products [3]. *M. bovis* infections have also been detected in wildlife and can have severe consequences for the ecosystem. Moreover, animal TB bears a zoonotic potential and is therefore of public health concern. Therefore, diagnosis of bovine TB is crucial for a proper control of disease transmission to animals and humans. To achieve that, a thorough knowledge of the available strains of *M. bovis* is required.

The identification of *M. bovis* by conventional bacteriological techniques is time consuming. Therefore, molecular identification techniques (spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis) have been developed and were proved to be quicker and reliable in discriminating *M. bovis* from the other *Mycobacterium tuberculosis* complex [4-11]. Repeated sequences were identified in the genome of *M. bovis* and *M. tuberculosis* and were used to type *M. bovis* by MIRU-VNTR analysis [4-11]. Therefore, we aimed at MIRU-VNTR analysis of cases of TB infection in local cattle in Egypt. Here, we applied MIRU-VNTR typing on samples collected from tuberculin-positive cattle in local farms in Damietta Province.

2. Materials and Methods

2.1. Sample Collection

Samples were collected from different cattle farms, in Damietta Province. Samples were obtained from cattle that showed positive results for tuberculin test. Sample collection was performed under the recommendations and approval of Mansoura University Animal Care and Use Committee.

2.1.1. Milk Samples

Fifty milk samples were collected from animals (in two cattle farms in Damietta Province) that showed positive results for tuberculin test and had mastitis. Five ml of milk were collected aseptically in sterile tubes.

2.1.2. Blood Samples

One hundred blood samples were collected from cattle (in three different farms in Damietta Province) that showed positive results for tuberculin test.

2.2. Isolation of Mycobacterium Species on Culture Medium

2.2.1. Preparation of Culture Media Used for Isolation (Middlebrook 7H10 Agar (BBL, Difco))

Nineteen grams of the agar powder were suspended in 900 ml of purified water containing 5 ml of glycerol and mixed thoroughly. The mixture was heated with frequent agitation and boiled for one minute to completely dissolve the powder. The mixture was autoclaved and then 100 ml of Middlebrook OADC Enrichment were added to the medium when cooled to 50-55°C.

2.2.2. Preparation of Milk Samples

Sample preparation was performed according to a previously published procedure [12]. Two ml of 4% H_2SO_4 were added to the sample and was incubated for 30 min at room temperature. Later, 16 ml of sterile distilled water were added to the previous mixture, and the whole mixture was centrifuged at 3000 rpm for 20 min. The supernatant was decanted into 5% phenol, and the sediment was used to make a direct smear, which was inoculated into two L-J medium slant (one contains 4% sodium pyruvate, and the other contains 5% glycerol) and was then incubated at 37°C for 3 weeks. Cultures were examined daily for one week and then once weekly for 6-8 weeks.

2.3. DNA Extraction from Blood Samples

DNA preparation from blood was performed by using DNA preparation Kit following the protocol of the manufacturer (Jenabioscience, Germany).

2.4. Confirmation of Mycobacterial Infection by Specific Gene Analysis

DNA, from blood samples collected from tuberculin-positive cattle farms, was tested for *IS6110*, *katG*, *gyrA*, *oxyR*, *pncA* genes (Table 1). Primer sequences for target genes were selected from previous literature [13] and were obtained from Metabion (Germany).

2.5. MIRU-VNTR Typing

MIRU-VNTR typing of DNA samples was performed using the primers shown in Table 1. Primer sequences for target loci were selected from previous reports [4,5,6]. Basically, the 20 µl PCR reaction consisted of 10 µl pf PCR mastermix (Intron Biotechnology, Korea), 1 µl (10 pmol) of each primer (forward or reverse), 2 µl of sample DNA and up to 20 µl nuclease-free water. The thermal cycling included 1 cycle of 12 min at 94°C, 40 cycles of 30 sec at 94°C, 1 min at the chosen annealing temperature according to the melting temperature of each primer sets (Table 1), 90 sec at 72°C, followed by 1 cycle of 7 min at 72°C. PCR products were resolved by electrophoresis using 1.5% agarose gels (in TAE buffer, Bioshop). The gel was placed in an electrophoresis tank containing 0.5% TBE and run for 1 h at 80 V. Markers (50 and 100 bp ladder, Qiagen and iNtTRON, respectively) were electrophoresed beside the DNA samples to measure the size of the DNA fragments. The DNA fragments were then visualised on a UV transilluminator using long wavelength ultraviolet light.

Table 1. Primer sequences, the expected product size for each used primer-set, and the appropriate annealing temperature used for PCR (Metabion) [4,5,6,13]

Target locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Ecpected Locus/ Repeat unit size (bp)	Annealing temperature (°C)
ETR-A	AAATCGGTCCCATCACCTTCTTAT	CGAAGCCTGGGGTGCCCGCGATTT	75	60
ETR-B	GCGAACACCAGGACAGCATCATG	GGCATGCCGGTGATCGAGTGG	57	60
ETR-C	GTGAGTCGCTGCAGAACCTGCAG	GGCGTCTTGACCTCCACGAGTG	58	60
ETR-D	CAGGTCACAACGAGAGGAAGAGC	GCGGATCGGCCAGCGACTCCTC	77	60
ETR-E	GCGGATCGGCCAGCGACTCCTC	CGGAACGCTGGTCACCACCTAAG	53	60
MIRU-10	GTTCTTGACCAACTGCAGTCGTCC	GCCACCTTGGTGATCAGCTACCT	53	58.8
MIRU-16	TCGGTGATCGGGTCCAGTCCAAGTA	CCCGTCGTGCAGCCCTGGTAC	53	58.8
MIRU-26	TAGGTCTACCGTCGAAATCTGTGAC	CATAGGCGACCAGGCGAATAG	51	58.8
MIRU-40	GGGTTGCTGGATGACAACGTGT	GGGTGATCTCGGCGAAATCAGATA	54	58.8
QUB-3232	CAGACCCGGCGTCATCAAC	CCAAGGGCGGCATTGTGTT	56	55
QUB-3336	ATCCCCGCGGTACCCATC	GCCAGCGGTGTCGACTATCC	59	55
QUB-1895	GGTGCACGGCCTCGGCTCC	AAGCCCCGCCGCCAATCAA	57	55
IS6110	CTCGTCCAGCGCCGCTTCGG	CCTGCGAGCGTAGGCGTCGG	123	68
katG	TCAGCCACGACCTCGTCGG	AGGCGGATGCGACCACCGTT	163	65
gyrA	CGAGACCATGGGCAACTACCA	CATTGCCTGGCGAGCCGAA	131	65
oxyR	CGCGCTGTCAGAGCTGACTTT	TCTGCGGAATCAGTGTCACC	150	60
pncA	ATCAGCGACTACCTGGCCGA	GATTGCCGACGTGTCCAGAC	180	66

2.6. Data Analysis

Allele frequency for the single MIRU-VNTR locus was calculated as previously stated [14], where the genetic profile or the frequency of each assigned copy number (number of certain copy number appearance in the selected population) was divided by the number of samples/isolates in the selected population. Furthermore, genetic diversity for the locus or allele diversity (h) was calculated as $h = 1 - \Sigma xi^2[n/(n - 1)]$, where xi is the frequency of the ith allele at the locus, Σxi^2 is the summation of all recorded (allele frequency)^2 for a single locus, n is the number of isolates in the sample, and $n/(n - 1)$ is a correction for bias in small samples [14].

3. Results

3.1. Mycobacterial Isolation

Milk samples (collected from farms that showed positive results for tuberculin test) did not show any positive result for mycobacterial isolation.

Table 2. The recorded copy number analysis of tested MIRU-VNTR loci in all tested samples and the results of specific gene analysis

Source	Sample	*IS6110*	*katG*	*gyrA*	*oxyR*	*pncA*	ETR-A copy number	ETR-B copy number	ETR-C copy number	ETR-D copy number	ETR-E copy number	MIRU-10 copy number	MIRU-16 copy number	MIRU-26 copy number	MIRU-40 copy number	QUB-3232 copy number	QUB-3336 copy number	QUB-1895 copy number
Farm A	Blood 1	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 2	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 3	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 4	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 5	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 6	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 7	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 8	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 9	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 10	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 11	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 12	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 13	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 14	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 15	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 16	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 17	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 18	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 19	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 20	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 21	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 22	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 23	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 24	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 25	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 26	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 27	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 28	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 29	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 30	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 31	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 32	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 33	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 34	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 35	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 36	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 37	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 38	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 39	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 40	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2

Source	Sample	*IS6110*	*katG*	*gyrA*	*oxyR*	*pncA*	ETR-A copy number	ETR-B copy number	ETR-C copy number	ETR-D copy number	ETR-E copy number	MIRU-10 copy number	MIRU-16 copy number	MIRU-26 copy number	MIRU-40 copy number	QUB-3232 copy number	QUB-3336 copy number	QUB-1895 copy number
Farm B	Blood 41	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 42	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 43	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 44	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 45	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 46	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 47	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 48	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 49	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 50	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 51	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 52	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 53	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 54	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 55	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 56	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 57	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 58	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 59	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 60	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 61	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 62	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 63	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 64	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 65	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 66	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 67	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 68	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 69	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 70	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
Farm C	Blood 71	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 72	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 73	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 74	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 75	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 76	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 77	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 78	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 79	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 80	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 81	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 82	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 83	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 84	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 85	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 86	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 87	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 88	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 89	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 90	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 91	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 92	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 93	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 94	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 95	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 96	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 97	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 98	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 99	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2
	Blood 100	+	+	+	+	+	75x1	57x1	58 x 1	77 x 1	53 x 2	53 x 1	53 x 1	51 x 1	54 x 1	56 x 2	59 x 2	57 x 2

3.2. Confirmation of Mycobacterial Infection by Specific Gene Analysis

DNA, extracted from blood samples (collected from tuberculin-positive cattle farms), was tested for *IS6110*, *katG*, *gyrA*, *oxyR*, *pncA* genes. All DNA samples were positive for all tested genes, and gave the expected PCR product sizes (Table 2).

3.3. MIRU-VNTR Typing

Although the selected MIRU-VNTR loci gave positive PCR result for their relative copy number (Table 1), none of the selected MIRU-VNTR loci showed polymorphism (in the copy number) across all studied samples.

3.4. Allelic Diversity of Target MIRU-VNTR Loci

The allelic diversity was calculated for selected MIRU-VNTR loci (Table 3). Due to the lack of polymorphism (in the MIRU-VNTR copy number), none of the selected MIRU-VNTR loci showed any discriminatory power in the genetic analysis of field samples.

Table 3. Calculating Allele frequency and genetic diversity (*h*) for each tested locus

Tandem Repeat locus	Number of alleles	Copy number	Allele Frequency	Allele diversity (*h*)
ETR-A	1	1	1	0
ETR-B	1	1	1	0
ETR-C	1	1	1	0
ETR-D	1	1	1	0
ETR-E	1	2	1	0
MIRU-10	1	1	1	0
MIRU-16	1	1	1	0
MIRU-26	1	1	1	0
MIRU-40	1	1	1	0
QUB-3232	1	2	1	0
QUB-3336	1	2	1	0
QUB-1895	1	2	1	0

4. Discussion

MIRU-VNTR is used as an approach to discriminate *Mycobacterium tuberculosis* complex [4-11]. In this study, we have accomplished MIRU-VNTR analysis in cases of bovine TB in Egypt, along with mycobacterial specific gene analysis (*IS6110*, *katG*, *gyrA*, *oxyR*, *pncA*).

Mycobacterial isolation failed from collected milk samples from positive cases of tuberculin test. Milk was previously recorded as a poor carrier for bovine TB infection, where researchers obtained poor percentage (10%) for bovine TB isolation from positive or suspected cases of TB infection [15].

All tested DNA samples, extracted from blood samples (collected from tuberculin-positive cattle farms), were positive for the selected mycobacterial genes, *IS6110*, *katG*, *gyrA*, *oxyR*, *pncA*, at the expected product size. A previous study performed on *M. bovis* in Germany [13], revealed the positivity of some *M. bovis*-positive, *IS6110*-positive samples to *katG*, *gyrA*, *oxyR*, and *pncA*. However, in the same study, other *M. bovis*-positive, *IS6110*-positive samples were only positive for *gyrA*. In the same study, *M. tuberculosis* samples lacked one, two, or three of the four investigated targets (*katG*, *gyrA*, *oxyR*, and *pncA*), and only one sample contained the four targets.

The MIRU-VNTR loci analysis did not show any discriminatory power for bovine TB genetic analysis. Our findings were close to researchers in New Zealand who worked on *M. bovis* MIRU-VNTR typing [11], where MIRU-10, MIRU-16, MIRU-40, QUB-3232, QUB-3336, and QUB-1895 did not have any discriminatory power for *Mycobacteria* typing, and ETR-C and ETR-D had medium and high discriminatory power for *Mycobacteria* typing, respectively. However, in their study, ETR-B and ETR-A had medium and high discriminatory powers for *Mycobacteria* typing, respectively, and ETR-E did not have any discriminatory power for *Mycobacteria* typing. Our study findings were also close to the data revealed by Korean researchers who worked on *M. bovis* MIRU-VNTR typing, where MIRU-10, MIRU-16, MIRU-40, and ETR-C had no discriminatory power for bovine TB typing in Korea [6]. In their study, MIRU-26, QUB-1895, and ETR-B had minimal discriminatory power for bovine TB typing. On the other hand, QUB-3232 and ETR-A had medium discriminatory power, and QUB-3336 had the highest discriminatory power across all tested loci. Furthermore, MIRU-VNTR typing of bovine TB in Brazil, caused by *M. bovis*, revealed the high discriminatory power of ETR-A, ETR-B, ETR-C, and MIRU-16, when compared to the minimal to nil discriminatory power of MIRU-26, MIRU-40, and MIRU-10 [7]. The lack of discriminatory power in our result could be attributed to the small geographical area our investigation included.

In comparison with molecular typing of *Mycobacterium tuberculosis*, some ETRs were found polymorphic in *Mycobacterium tuberculosis* strains by other researchers in Thailand [9], where high discriminatory power for human TB typing was recorded by using ETR-B, ETR-D, and ETR-E. In contrast to our findings, where MIRUs did not have any discriminatory power, their work showed another high discriminatory power for TB typing by using MIRU-10, MIRU-16, and MIRU-26.

5. Conclusion

In the current study, we were able to perform MIRU-VNTR loci analysis for TB-infected cattle in Egypt. However, the MIRU-VNTR loci analysis did not any discriminatory power for bovine TB genetic analysis. Therefore, further analysis is required for clear distinction between field isolates/samples, either through using other MIRU-VNTR loci or through spligotyping.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

Project design was performed by HM and MS. Sample collection was achieved by HM, SA, YA, and WA. Microbial isolation was performed by collaboration between A. Ammar, A. Amer, and EM. MIRU-VNTR typing was performed by collaboration between HM, MS, and ME. Mycobacterial gene-specific analysis and project data analysis were accomplished by HM.

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