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Molecular Characteristics of *Mycobacterium bovis* in Correlation with the Spread of Bovinetuberculosis (BTB) on a Dairy Farm

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Abstract: Bovine tuberculos is an important zoonotic disease that spread through out the world. *Mycobacterium bovis (M.bovis)* is the causative agent of bovine tuberculosis in cattle, other domestic animals and wildlife. *M.bovis* potentially causing health hazards in both animals and humans. This aims of the study were to determine the molecular characteristics of *M.bovis* bacteria and correlation with the spread of bovine tuberculosis(btb) in a dairy farm in Enrekang district, South Sulawesi province.This study was exploratory study to examine the properties of *M.bovis* and genotyping with Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) technique on sixty milk samples from dairy cows at in Enrekang district, South Sulawesi province. Results showed 6 dairy cow milk samples positive for *M.bovis* by multiplex PCR technique with amplicon length of 500bp. Further more, 6 milk samples of dairy cows positive for *M.bovis* done molecular characteristics with MIRU-VNTR technique. Based on MIRU-VNTR technique, *M.bovis* which causes bovine tuberculosis in Enrekang district, South Sulawesi province were identical with *M.bovis* strain(1290/03).

Key words: *Mycobacterium bovis* · Dairy Cow · *Bovine tuberculosis* · MIRU-VNTR

INTRODUCTION

Bovine tuberculosis (btb) caused by *Mycobacterium bovis (M.bovis)* is a zoonotic disease with a complex epidemiological pattern. Bovine tuberculosis has an extraordinarily broad mammalian host range that includes cattle, domestic livestock and wildlife [1]. *M.bovis* is a member of the Mycobacterium tuberculosis complex, a group that includes also *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium microti* [2-4].

Transmission can occur within and between farm animal and wildlife populations, also to human. Human most often infected by *M.bovis* through contaminated food or water, unpasteurized dairy products, direct contact with a wound, or by inhaling airborne bacteria exhaled by animals infected with *M.bovis* [5-7]. *M.bovis* infection spreads to livestock primarily through aerosols either from coughing or sneezing or animals with open tuberculosis from infected dust particles. Considering the dominance of aerosol transmission, the infection can spread more rapidly in an intensive dairy farm models/caged. Aerosol transmission is effective only at shorter distances (1-2 m) and hence the density of livestock is as significant factor in determining the rate of transmission [8].

Diagnosis is important to control bovine tuberculosis (btb) and to help prevent transmission Tests that can be performed to identify *M. bovis* are microscopic examination by staining acid-fast bacilli (AFB), culture, PCR (Polymerase Chain Reaction) [9], blood tests {Gamma-Interferon assay (IFN□ assay), lymphocyte proliferation assay and Enzyme-Linked Immunosorbent Assay (ELISA), tuberculin skin test and histological test [1, 4, 10, 11].

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To control bovine tuberculosis (btb), it is necessary to know the number and types of *M.bovis* strains existing in the field. The advent of molecular techniques has greatly contributed to the identification and typing of *M.bovis*.The molecular techniques also enable the of *M.bovis* in a short time. Moreover, the molecular typing method can distinguish *M.bovis* from other *M. tuberculosis complex* and it can discriminate between clinical *M.bovis* Isolates. Genotyping methods, such as spoligotyping and MIRU-VNTR, applied to clinical strains have proven to be valuable tools for btb epidemiological studies and disease control. This epidemiological information is useful for tracing the outbreaks and transmission among domestic or wild animals [12, 13].

Data from the Office International des Epizooties (OIE) showed that in Indonesia, including the province of South Sulawesi, clinically never reported any cases of *bovine tuberculosis*. Detecting sero-prevalence using ELISA method at Regency Bangli, Bali province, point out 2,22% BTB'S positive cows [14]. Detection by conventional methods need long time, its accuracy is low with frequent happening of contaminating bacteria [15]. The objective of this paper is to characterize *M.bovis* in dairy cattle at Enrekang, South Sulawesi, Indonesia.

MATERIAL AND METHODS

Study Collection: The research was conducted in dairy farm at Enrekang regency for sample selection and the Laboratory Immunology and Molecular Biology of Hasanuddin University for MIRU-VNTR analysis. Six samples of milk that positive were *M.bovis* by analysis of multiplex PCR were included in this study (preliminary study).

DNA Preparation: DNA extraction of *M.bovis* from milk samples using Sonication and boiling methods [16]. A total of 1 ml of milk decontamination results inserted into eppendorf tubes. Subsequently centrifuged at 13,000 rpm for 15 minutes. The supernatant was removed and added to 100 ul of sterile water (ddH_2O). Eppendorf tubes containing isolates were then inserted into the water bath (temperature 95^0C) for 25 minutes, then sonicated for 20 minutes and then centrifuged 13.000 rpm for 15 minutes. Then the supernatant containing the DNA was transferred to a new tube. After completion of DNA extraction the DNA stored be stored at -20^0C (if not directly in thePCR).

Amplification-PCR (Genotyping MIRU-VNTR): This procedure was done on DNA samples, the DNA extracts of *M.bovis* as a positive control and distilled water as a negative control. "PCR mix" inserted into PCR tube,PCR Mix with the composition.

Amplification was performed using PCR machine (DNA Thermal Cycler) [17]. The initial phase of denaturation at 95°C for 15 minutes, the next 94°C for 1 min, annealing at a temperature of 59°C for 1 min, extension 72°C for 1.5 min by 40 cycles followed by a final extension temperature of 72°C for 10 min and 4°C, 30 min for storage Preparation using 2% agarose. [18-20].

Data Analysis and Identification of Strain: PCR fragment size was determined from the position of DNA size marker (Marker) and fragment sizes *M.bovis* and M.bovis strains as controls number of repetitions were estimated using a scoring table MIRU-VNTR allelic. This table shows the correspondence between the expected PCR fragment size and MIRU-VNTR allelic scores for the 24 MIRU-VNTR polymorphic loci. Genotype is expressed as a numeric code representing the number MIRU-VNTRs in each of the 24 loci. Numerical code then used to identify strains of *M.bovis* by sending data to an online data base MIRU-VNTRplus [21].

RESULTS

Milk samples of dairy cows positive for *M.bovis* done molecular characteristic with MIRU-VNTR Techniques showed that this sample closely related with *M.bovis* that have been found in several other countries, but for this Makassar strain has a slightly different pattern of strain is indicated by the presence of the dividing line of the other strains *M.bovis* dendogram.

Dendogram (Figure 1) showed that isolates MKS-ALI1, MKS-L2, MKS-SUN1, MKS-NAR2, MKS-SUN3 and MKS-NAS1 from Enrekang district South-Sulawesi province overall isolates still in one group and overall isolates from Enrekang district South-Sulawesi province was in a group with isolates from bovis strain (1290/03).

Having obtained dendrogram, then madekofenetik correlation analysis. This analysis aims to show the relationship similarity between microbial strains tested. The most common method used is the Unweighted Pair Group Method with Averages/UPGMA [22]. The results showed a kofenotik correlation coefficient =0.99884, showed that six positive milk samples *M.bovis* have acceptable closeness.

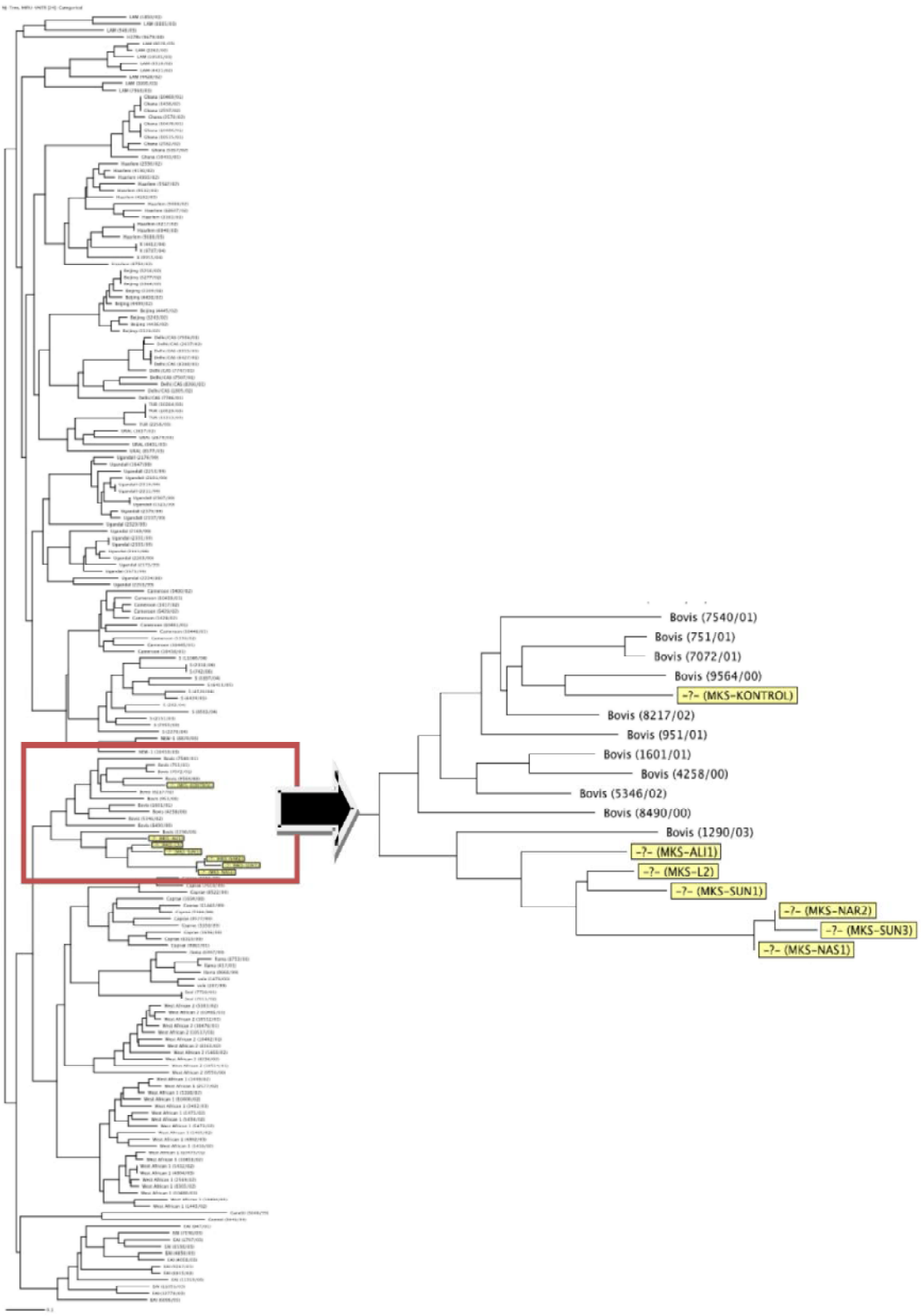

Fig. 1: Dendogram of globalM.bovis genotypes

Table 1: PCR Mix with the composition

Loci	Group A (µl)	Group B (µl)	Group C (µl)	Group D (µl)
ddH2O	9.1	9.9	10.3	9.5
10X PCR buffer	2	2	2	2
5X Q solution	4	4	4	4
25 mM $MgCl_2$	1.2	0.4	0	0.8
5 mMdNTP	0.8	0.8	0.8	0.8
Reverse primer (20pmol)	0.4	0.4	0.4	0.4
Forward primer (20pmol)	0.4	0.4	0.4	0.4
Hotstart DNA pol.	0.08	0.08	0.08	0.08
DNA sample (1 ng/µl)	2	2	2	2
Total premix	20	20	20	20

Notes: Primer division based on the volume of $MgCl_2$: Group A consists of: MIRU04, MIRU26, MIRU40, Mtub30, Mtub39 and QUB4156. Group B: MIRU10, MIRU16, MIRU31, Mtub29, ETRB and Mtub34. Group C: MIRU20, MIRU24, MIRU27, Mtub04, ETRC, ETRA, QUB11b, Mtub21 and QUB26. Group D: MIRU02, MIRU23 and MIRU39.

Table 2: Primer sequences and the size of repeat units of the VNTR locus in this study

Locus	PCR primer Sequences (5'-3')	Repeat unit size
MIRU-4-F	GCGCGAGAGCCCGAACTGC	19 bp
MIRU-4-R	GCGCAGCAGAAACGCCAGC	19 bp
MIRU-26F	TAGGTCTACCGTCGAAATCTGTGAC	25 bp
MIRU-26R	CATAGGCGACCAGGCGAATAG	21 bp
MIRU-40F	GGGTTGCTGGATGACAACGTGT	22 bp
MIRU-40R	GGGTGATCTCGGCGAAATCAGATA	24 bp
MIRU-10F	GTTCTTGACCAACTGCAGTCGTCC	24 bp
MIRU-10R	GCCACCTTGGTGATCAGCTACCT	23 bp
MIRU-16F	TCGCTGATCGGGTCCAGTCCAAGTA	25 bp
MIRU-16R	CCCGTCGTGCAGCCCTGGTAC	21 bp
MIRU-31F	ACTGATTGGCTTCATACGGCTTTA	24 bp
MIRU-31R	GTGCCGACGTGGTCTTGAT	19 bp
ETR A-F	AAATCGGTCCCATCACCTTCTTAT	24 bp
ETR A-R	CGAAGCCTGGGGTGCCCGCGATTT	24 bp
QUB-11b F	CGTAAGGGGGATGCGGGAAATAGG	24 bp
QUB-11b R	CGAAGTGAATGGTGGCAT	18 bp
QUB-26 F	AACGCTCAGCTGTCGGAT	18 bp
QUB-26 R	CGGCCGTGCCGGCCAGGT CCTTCCCGAT	28 bp
MIRU-2F	GGACTTGCAGCAATGGACCAACT	24 bp
MIRU-2R	TACTCGGACGCCGGCTCAAAAT	22 bp
MIRU-23F	CTGTCGATGGCCGCAACAAAACG	23 bp
MIRU-23R	AGCTCAACGGGTTCGCCCTTTTGTC	25 bp
MIRU-39F	CGCATCGACAAACTGGAGCCAAAC	24 bp
MIRU-39R	CGGAAACGTCTACGCCCCACACAT	24 bp
MIRU-20F	TCGGAGAGATGCCCTTCGAGTTAG	24 bp
MIRU-20R	GGAGACCGCGACCAGGTACTTGTA	24 bp
MIRU-24F	CGACCAAGATGTGCAGGAATACAT	24 bp
MIRU-24R	GGGCGAGTTGAGCTCACAGAA	21 bp
MIRU-27F	TCGAAAGCCTCTGCGTGCCAGTAA	24 bp
MIRU-27R	GCGATGTGAGCGTGCCACTCAA	22 bp

Table 3: VNTR analysis of the *M. bovis* isolates

	MIRU												ETR			QUB			Mtub				
Isolates	02	04	40	10	16	20	23	24	26	27	31	39	A	B	C	26	4156	04	21	29	30	34	39
ALI	1	2	3	2	2	2	3	5	2	4	3	3	2	2	5	5	4	2	2	2	4	4	2
L2	2	3	2	3	2	3	6	2	4	3	3	2	7	5	5	4	2	2	2	3	4	3	2
NAR2	3	6		3																			
NAS1	3	6		3																			
SUN1	2	3	2	3	2	3	6	2	4	3	2	2	2	5	5	4	2	2	2	3	4	2	2
SUN3	3																						

DISCUSSION

This study examines the 60 milk samples of dairy cattle farms in Enrekang district South Sulawesi province. Infected milk is a source of disease transmission to calves, pigs and humans. Approximately 5% of cows suffering fromTB infection showed inflammation of the udder (tuberculous mastitis). At the beginning of the disease process, even though it contains the TB bacili, appear physically normal milk. Further changes include changes in color and the quality is declining. The milk becomes more watery and does not contain coconut milk (cream). At the level of disease, more milk changes significantly appears with the presence of the clumps that are purulent [23]. Factors that contribute to infection in cattle is the frequency of contact with humans is very high, cage distance is very close to population centers, adverse environmental conditions such as high humidity, poor ventilation cage and the poor feed condition [6]. In addition, knowledge of farmers about infectious agents and the preventive measures is very limited. Clearly better information to inform farmers about causes, consequences and risk of infection is needed.

This study shows that out of milk samples from 60 dairy cows, there are 6 samples (10%) were positive *M. Bovis* by Multiplex PCR test using specific primers on amplicon length of 500 bp (The preliminary study). Milk components do not inhibit the PCR reaction [24].

Six samples that have positive PCR is then performed *M. Bovis* genotyping with MIRU-VNTR techniques to know the characteristics of *M.bovis* that is associated with the spread of bovine tuberculosis incidence in Enrekang district, South Sulawesi province.

Polymerase Chain Reaction based genotyping using MIRU-VNTR techniques in *M. Bovis* isolates. The MIRU-VNTR technique has proved a valuable tool for further development and application of epidemiological research to identify the sources of infection and achieve a wider knowledge of bovine tuberculosis transmission routes [25, 26]. Previous studies already demonstrated the efficiency of the used methods in several countries [19, 26-28].

Genotyping by comparing isolates globally by sending data to MIRU-VNTRplus online database [21], MKS-ALI1, MKS-L2, MKS-SUN1, MKS-NAR2, MKS-SUN3 and MKS-NAS1 isolates have similarities with strains bovis isolates (1290/03). The results of the study isolates from Enrekang district, South Sulawesi province still in the group. It shows the incidence of bovine tuberculosis in Enrekang caused by the same bacteria despite having diversity at some loci.

CONCLUSION

Molecular characteristics of *M.bovis* by MIRU-VNTR technique related to the spread of bovine tuberculosis incidence in dairy cattle in Enrekang district, South Sulawesi province Based MIRU-VNTR technique, *M.bovis* which causes bovine tuberculosis incidence in Enrekang district identical with bovis strain (1290/03).

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