Journal of Agricultural Science and Technology B 4 (2014) 215-223
Earlier title: Journal of Agricultural Science and Technology, ISSN 1939-1250

Nontuberculous Mycobacteria Infections in Katavi Rukwa Ecosystems

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Received: January 24, 2014 / Published: March 20, 2014.

Abstract: A study on nontuberculous mycobacteria (NTM) was carried out in wildlife-livestock interface of Katavi Rukwa ecosystem (KRE). 328 livestock tissues and 178 wild animals were cultured, wild animals were sampled opportunistically during professional hunting and game cropping operations in the KRE protected areas. The objective of the study was to generate data on epidemiology of NTM in the wildlife-livestock interface of the KRE. Methods used to identify the NTM were: culture and isolation, polymerase chain reaction, protein heat shock 65 kilodalton (*hsp65*) and sequencing. Mycobacteria were detected on 25.9% and 11.9% of livestock and wildlife tissue cultures, respectively. The most NTM isolated were *M. kansasii* (30%), *M. gastri* (30%), *M. fortuitum* (1%), *M. intracellulare* (4%), *M. indicus pranii* (4%), *M. nonchromogenicum* (6%) and *M. lentiflavum* (6%). Other NTM in smaller percentages were *M. hibernae*, *M. engbaekii*, *M. septicum*, *M. arupense* and *M. godii*. Due to rise of NTM infection in both human and animals, it is recommended that awareness and laboratory facilities be improved to curb the underreporting especially in TB-endemic countries. For species specific identification, a network of national and regional laboratories is promoted.

Key words: Mycobacteria, polymerase chain reaction, protein heat shock and sequencing.

Nomenclature

NTM Nontuberculous mycobactria
hsp65 Heat shock protein 65 kilodalton
KRE Katavi Rukwa Ecosystem

1. Introduction

Recently, there has been an increase in disease caused by organisms broadly categorized as nontuberculous mycobacteria (NTM), a generic term for mycobacteria not included in the *Mycobacterium tuberculosis* complex and other than *M. leprae*. Of these NTM, *Mycobacterium avium* complex (MAC) species are the most common cause of human and animal disease globally [1, 2]. Other NTM of importance are *M. intracellurare* and *M. fortuitum* [3]. NTM are naturally resistant to most anti-tuberculosis drugs [4] and recently to the infections, due to NTM are growing steadily around the world. The clinical relevance of the MAC in humans has been amplified in recent decades with the increasing population of immunocompromised individuals and the AIDS pandemic [5]. Other studies detected a wide range of potentially pathogenic NTM from the environment that suggests drinking untreated water and living in close contact with cattle or other domestic animals may be a risk factors associated with the possibility of humans and animals acquiring NTM infections from these ecosystems [6, 7]. A microbial danger to human and animal health due to the NTM is on a rise [8] because most of them are inherently resistant or

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partially susceptible to the standard anti-tubercular drugs [9]. NTM can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease or disseminated disease [10]. Mycobacteria other than *M. tuberculosis* and *M. leprae* are generally free-living organisms that are ubiquitous in the environment [11]. They have been recovered from surface water, tap water, soil, domestic and wild animals, milk and food products [12]. *Mycobacterium avium* complex infections in HIV-infected patients and NTM infections in solid organ transplant candidates and recipients are also discussed elsewhere [7]. The diagnosis of diseases caused by NTM is difficult, because NTM are prevalent in the environment such as soil and water, and because they have fastidious properties. In this study we investigated isolates of NTM for their distribution pattern and accurate species identification in Katavi ecosystem by sequencing the molecular isolates.

Studies show that nontuberculous mycobacteria are more common than *M. tuberculosis* in adenitis tuberculosis diagnosed case in which HIV infection and raw animal products are among the risk factors identified for both *M. bovis* and nontuberculous mycobacterial adenitis [13].

The objectives for this study were to determine presence of NTM in wildlife and livestock tissues, molecular characterization and determination of genetic spatial-temporal variation of disease in Katavi Rukwa ecosystem (KRE), Tanzania.

2. Methods

2.1 Ethics and Consent for Livestock Sampling

This study was approved by Sokoine University of Agriculture (SUA/ADM/R.1/8/NKASI/MPANDA/ 2009) and accepted by the respective district council in the study area. The animal owners were requested and upon consent the sampling was carried out. Tanzania Wildlife Research Institute (TAWIRI) and Tanzania National Parks (TANAPA) approved research on wild animals at KRE (TAWIRI/TANAPA/2009).

2.2 Study Area

The fieldwork was carried out in two districts Mpanda and Nkasi within Rukwa region (Mpanda newly formed Katavi region). Nkasi district is located South West of Tanzania between latitudes 6°58′ and 8°17′ South of Equator and longitude 30°20′ and 31°30′ East of Greenwich. The district is bordered by Mpanda District to the North, Sumbawanga District and Zambia to the South, Sumbawanga Municipal to the East and to the West by the Democratic Republic of Congo through Lake Tanganyika. Nkasi District with an area of 13,124 km^2 has a human population of about 228,885. Katavi Region was created in March 2012, replacing the district formerly known as Mpanda that lies between latitudes 5°15′ and 7°03′ South and longitude 30°02′ and 33°31′ East (Fig. 1).

All livestock samples were obtained from abattoirs and opportunistically from hunter killed wild animals. The objective of this test method was to identify *Mycobacteria* to the species level by sequencing the first 500 base pairs of the 16S ribosomal gene. The genomic DNA is extracted from bacterial cells grown or cultured in Lowensen Jensen media (LJM) in Glycerol (G) or Pyruvate (P). According to Berg [14] the 16S rDNA fragment of interest is amplified by polymerase chain reaction (PCR) and purified. Cycle sequencing is performed on the purified PCR product, labeling the appropriate nucleotide with one of four fluorescent tags (dye terminators). After the extension product is purified using a gel filtration cartridge, electrophoresis is performed on a genetic analyzer. The resulting forward and reverse sequences are aligned and edited to resolve base pair ambiguities in the consensus sequence using the MicroSEQ software from Applied Biosystems™. The edited consensus sequence is compared to a sequence database library revealing a list of close matches. The final species identification is determined based on the percent divergence from the compared sequences in the library. All bacterial genomes studied to date contain a unique pattern of repetitive sequences that, when

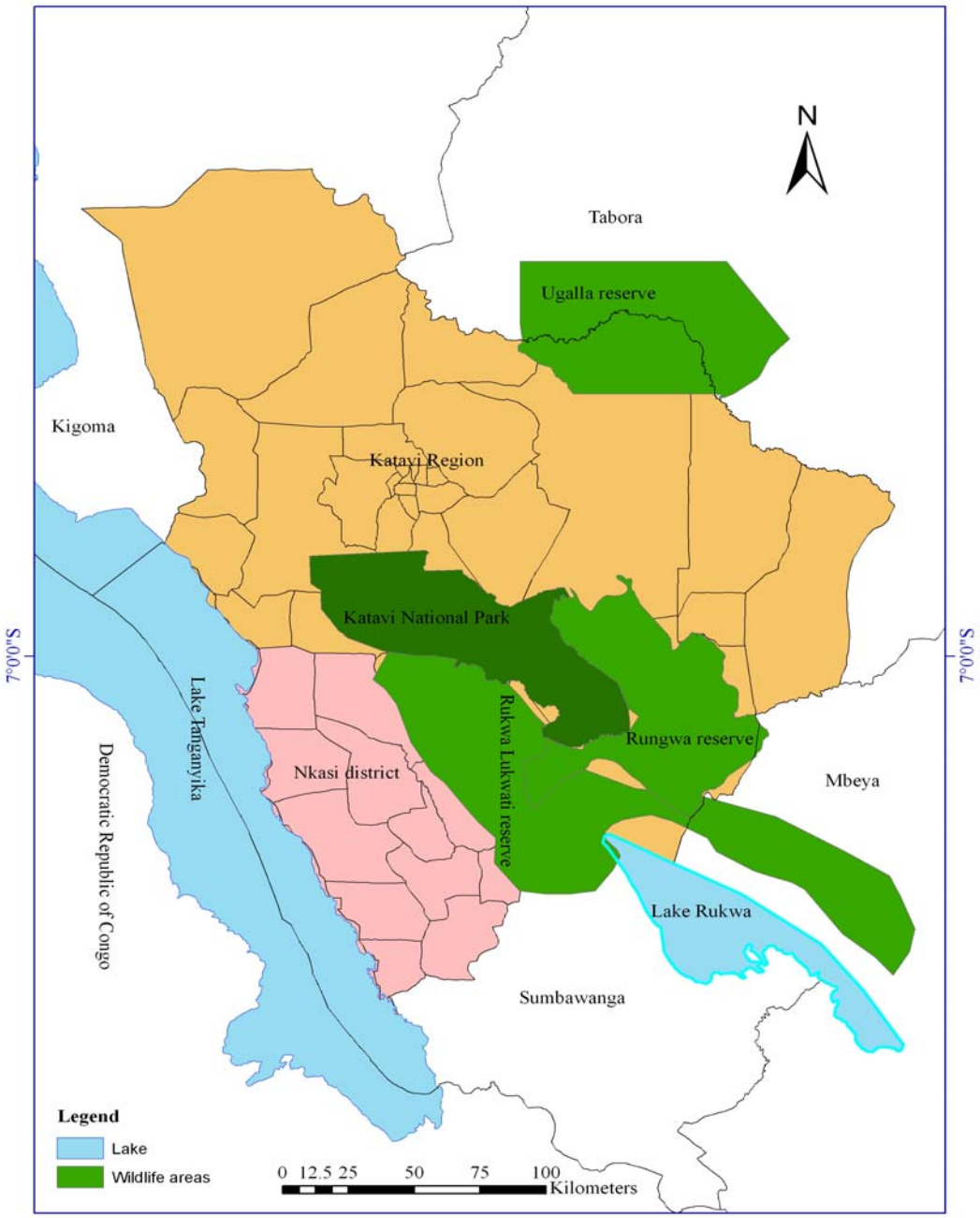

Fig. 1 A study area where samples from livestock and wild animal were collected.

amplified, can be used to differentiate between species and strains. This fingerprinting system amplifies the repetitive elements from a pure culture using a repetitive PCR method and then separates the amplicons by size. This creates a distinct DNA fingerprint that can be used for identification and strain typing. Principally mycobacterial pure colonies were added to nuclease free water and heat killed at 80 °C water bath temperature. Mycogenous PCR was performed according to previous study [14]. The 16S sequences were accepted for analysis if they had a readable length of 500 bp and more to cover the hypervariable signature regions in the 16S rDNA sequence [14, 15]. The sequencing primer 271 was used. Identity was ≥ 99% [15]. *hsp65* gene sequencing was used to differentiate these species according to

Richter et al. [16].

Mycobacteria isolates were identified to the species level by direct sequencing of fragments of 16S rDNA. To advance the identification of NTM species, a partial sequencing of the 65 kilodalton heat shock protein (*hsp65*) gene was used and then identified by comparing unknown sequences to reference databases by a GenBank NCBI FASTA BLAST search [16, 17]. This reaction amplified a 439-bp fragment within the *hsp65* gene using the primers Tb11 (5-ACCAACGATGGTGTGTCCAT-3) and Tb12 (5-CTTGTCGAACCGCATACCCT-3) [17]. All *Mycobacterium intracellulare* were confirmed by *hsp65* gene. Other mycobacterium such as *M. kansasii* and *M. gastri* were also identified by these sequencing.

3. Data Analysis

Quantitative data collected from the field and sampling were recorded and coded in the Microsoft Excel 5.0, and exported to EpiInfo® Version 3.1 (CDC, 2009) for Windows. The analysis in the EpiInfo® involved frequency, cross tabulation, mean, standard deviation and related statistics that were obtained and used to describe the characteristics and trends of variables. Arc GIS 9.3® in couple with SaTScan™ softwares was used to analyse spatial and temporal patterns of the disease both in the wildlife and livestock.

Obtained sequences of nontuberculous mycobacteria were edited and analyzed and sequences were compared to available sequences in GenBank by the NCBI blast sequence alignment tool. The isolates were determined to species based on the maximum score and maximum identity values on NCBI Blast alignment, a maximum score and maximum identity of ≥ 99% were accepted.

4. Results

Out of 506 tissue samples collected, all were inoculated. Only 102 inoculated cultures grown into media. Culture growths in the LJM-G were 78 samples and 82 samples in LJM-P. Among these, 23 cultures were found to grow in only one of the media. Twenty four samples were acid fast bacteria (AFB) negative. Eighty eight samples were AFB positives, among them only five members of mycobacterium tuberculosis complex (MTC) were detected by molecular methods and the rest were NTM. A detail from each livestock sample is presented in Table 1.

4.1 Nontuberculous Mycobacteria in Livestock

Unusual isolation was demonstrated in samples collected from the study area. Many samples from cattle and goats were culture positive, and the growth had number of AFB positives. Mycogenus PCR detected 25.3% NTM of all tissue samples cultured. Heat-killed AFB positive samples were investigated by mycogenus PCR for the presence or absence of the MTC. Only DNA from AFB positives colony were harvested, heat killed and tested for mycogenus PCR. Different and specific oligonucleotides primers were used in mycogenus PCR as previously described. All mycobacteria exhibited band 1,030 bp except those which belongs to MTC had two bands, the first 1,030 bp and 372 bp in addition (Fig. 2) that confirms a member of MTC. Five MTC strains detected were collected at abattoir from cattle originating in different parts of the study districts. All four MTC were from Katavi region and only one strain was collected from Nkasi district.

All these NTM were sequenced and identified to species level. Table 2 shows the different NTM identified to species level.

4.2 Nontuberculous Mycobacteria in Wild Animals

One hundred and eleven out of One hundred and nineteen tissue samples from different animal species inoculated into culture media showed growth. Thirty two tissue samples were confirmed AFB positive by microscopy. DNA from these 32 isolates was submitted to SUA tuberculosis laboratory for mycogenus

Table 1 Livestock tissue samples cultured and the microscopy identification.

Factors		Samples collected	LJM-G growth	LJM-P growth	Cocci	Mycobacteria	AFB + (P)	AFB + (G)
Sex	Female	63	11	12	3	15	9	11
	Male	264	67	70	12	73	56	65
Species	Bovine	294	78	82	15	85	65	76
	Caprine	33	3	3	0	3	3	3
Category	Bull	253	67	70	2	73	56	65
	Cow	43	11	12	8	13	9	11
	Calves	31	3	5	5	2	2	2
Status	Health	315	72	76	14	86	59	70
	Sick	2	1	1	0	0	1	1
	Weak	4	1	1	1	2	1	1
	Emaciated	5	4	4	0	0	4	4
Specimen	Liver	2	0	1	1	0	0	0
	Mediastinal	318	75	80	13	84	62	74
	Retropharyngeal	6	3	2	0	4	3	2
	Prescapular	1	0	1	2	0	0	0

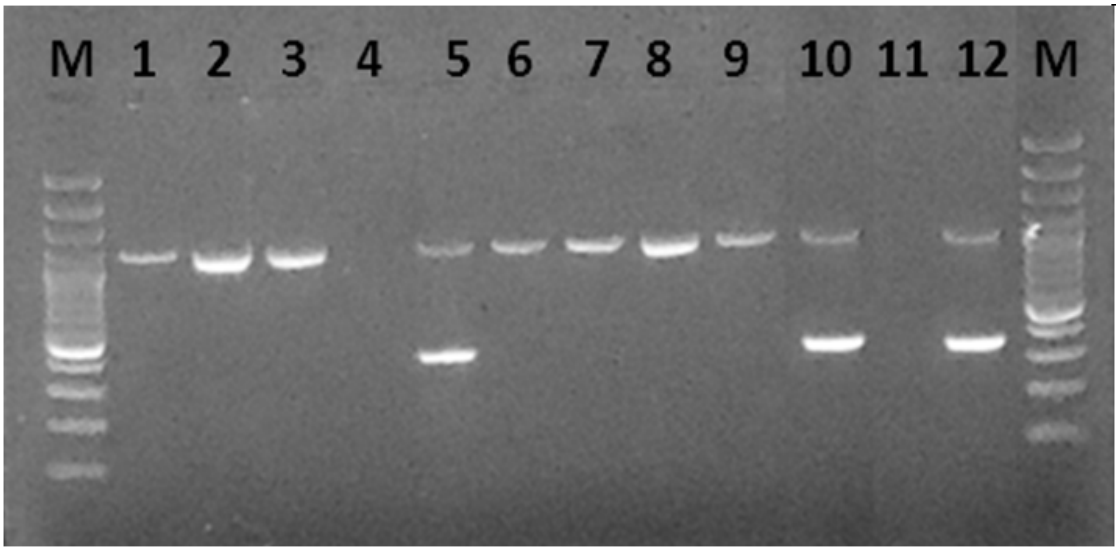

Fig. 2 PCR products by mycogenus typing of mycobacteria isolates from cattle: MAKER, S1 MP189, S2 MP183, S3 MP112, S4 MP31, S5 MP224, S6 MP43, S7 MP93, S8 MP58, S9 MP110, S10 *M. tuberculosis*, S11 water, S12 *M. bovis* ladder.

PCR analysis. Only 15 NTM out of 32 isolates (Table 3) were identified by 16S rDNA sequence analysis to species level. The species detected were grouped into the following: *Mycobacterium lentiflavum*, *Mycobacterium fortuitum* and *Mycobacterium peregrinum*. *hsp65* returned the same species in group.

The most nontuberculous mycobacteria isolated were *M. kansasii* (30%), *M. gastri* (30%), *M. fortuitum* (1%), *M. intracellulare* (4%), *M. indicus pranii* (4%), *M. nonchromogenicum* (6%) and *M. lentiflavum* (6%). Other NTM in smaller percentages were *M. hibernae*, *M. engbaekii*, *M. septicum*, *M. arupense* and *M. godii*. In livestock the isolates were *M. peregrinum*, *M. kansasii*, *M. gastri*, *M. nonchromogenicum*, *M. palustre*, *M. lentiflavum*, *M. goodie*, *M. moriokaense*, *M. smegmatis* and *M. intracellulare*.

In wild animals the same *M. fortuitum* isolate was found and *M. peregrinum* was isolated too. In case of the five samples (SUA No.: 152, 15, 13, 74 and 94) which were identified to be *M. kansasii*, *hsp65*

Table 2 Selected NTM isolated from Katavi cattle and goats in 2010 to 2012.

Ser No.	SUA ID	FLI ID	NTM species
1	MP502	12MA1251	*M. peregrinum*
2	MP26	12MA1252	*M. kansasii* and *gastri*
3	MPZ35	12MA1259	*M. nonchromogenicum*
4	MP243	12MA1261	*M. palustre*
5	MP35	12MA1263	*M. kansasii* and *gastri*
6	MP121	12MA1269	*M. lentiflavum*
7	MP83	12MA1293	*M. kansasii* and *gastri*
8	MP107	12MA1288	*M. kansasii* and *gastri*
9	MP91	12MA1294	*M. goodie*, *M. moriokaens*, *M. smegmatis*
10	MP63	12MA1298	*M. kansasii* and *gastri*
11	MP146	12MA1301	*M. kansasii* and *gastri*
12	MP166	12MA1304	*M. kansasii* and *gastri*
13	MP170	12MA1310	*M. intracellulare*
14	MP53	12MA1313	*M. kansasii* and *gastri*
15	MP30	12MA1315	*M. kansasii* and *gastri*
16	MP167	12MA1319	*M. lentiflavum*
17	MP231	12MA1255	*M. nonchromogenicum*
18	MPZ135	12MA1260	*M. indicus pranii* and *M. intracellulare*
19	MP227	12MA1292	*M. hibernae* and *M. engbaekii*
20	MP502	12MA1250	*Mycobacterium fortuitum*, *M. porcinum*
21	MP26	12MA1251	*Mycobacterium peregrinum*, *M. septicum*
22	MP243	12MA1261	*Mycobacterium intracellulare*, *M. indicus pranii*
23	MP183	12MA1262	*Mycobacterium kansasii*, *M. gastri*
24	MP121	12MA1269	*Mycobacterium lentiflavum*
25	MP33	12MA1270	*Mycobacterium kansasii*, *M. gastri*
26	MP17	12MA1297	*Mycobacterium kansasii*, *M. gastri*

Table 3 Frequency of culture isolation of nontuberculous mycobacteria.

Species	Number collected	AFB positive	NTM prevalence
Leopard (*Panthera pardus*)	5	4	3/4 (*M. lentiflavum*)
Buffaloes (*Syncerus caffer*)	52	13	5/13 (*M. fortuitum* and *M. peregrinum*)
Kudu (*Tragelaphus strepsiceros*)	1	0	0
Impala (*Aepyceros melampus*)	4	0	0
Hartebeest (*Alcelaphus buselaphus*)	10	2	1/2 (*M. fortuitum*)
Lion (*Panthera leo*)	12	4	3/4 (*M. fortuitum* and *M. peregrinum*)
Zebra (*Equus burchelli*)	7	2	0
Eland (*Taurotragus oryx*)	2	2	0
Baboon (*Papio anubis*)	2	0	0
Warthog (*Phacochoerus africanus*)	7	1	0
Bushpig (*Potamochoerus larvatus*)	2	1	0
Hippopotamus (*Hippopotamus amphibius*)	7	0	0
Reedbuck (*Redunca arundinum*)	2	0	0
Waterbuck (*Kobus ellipsiprymnus*)	2	1	1/2 (*M. fortuitum*)
Elephant (*Loxodonta africana*)	1	0	0
Crocodile (*Crocodylus niloticus*)	1	0	0
Oribi (*Ourebia ourebi*)	2	2	2/2 (*M. fortuitum*)

NTM from wildlife samples in KRE in 2010 to 2012.

determined the subspecies which is subspecies VI. The isolate 94 was identified as suspected mix of *M. kansasii* subspecies VI. The subspecies of all these isolates was determined by sequencing the *hsp65* gene and the determination was done according to Refs. [16, 18]. When sequencing the 16S rRNA gene, it was not able to discriminate firmly between *M. kansasii* and *M. gastri*. This was successfully done by sequencing the *hsp65* gene that determined them to subspecies. For the *hsp65* gene again, we analyzed only sequences which were at least 400 bp readable.

5. Discussion

Several nontuberculous mycobacteria species were isolated from livestock and wildlife in the current study, although these species may not be a true respiratory pathogen, but may demonstrate clinical disease in ungulate species [18] and extrapulmonary disease in immunocompromised humans [6, 19]. Percentage of isolation was relatively higher if compared to human isolate done in China [6, 19] from that in livestock [20]. *M. fortuitum* has been reported to be danger [19] though neglected as well as other important NTM infections [20, 21]. Several NTM species are now causing disease in human [22]. *M. peregrinum* in most studies was demonstrated as a group of *M. fortuitum* that frequently cause infection [6, 19]. Some NTM cause pulmonary infections that pose substantial difficulties with regard to clinical management and multi drug resistance [23]. Generally, most of nontuberculous mycobacteria are considered non-pathogenic in most species, but recent years these mycobacterial species have been associated with granulomatous lesions in both cattle and humans [7, 24].

M. kansasii is the most frequently cause of lung infections and its prevalence in the world has increased [25]. It can cause severe tuberculosis-like disease in humans and it has shown to cause skin test positive reactions in cattle [26-29]. *M. fortuitum* may also cause post-surgical infections [19, 29]. NTM have shown potential of forming biofilm that complicate treatment [28]. The above fact shows importance of these isolated NTM that are frequently increasing and feared to regularly infect human through consuming raw milk [30, 31]. The identification rate by sequencing of 16S rRNA and *hsp65* were 80% and 42%, respectively. The *hsp65* gene was less efficient than 16S rRNA for the identification of NTM by sequencing.

6. Conclusions

This study has demonstrated isolates of many NTM in both livestock and wildlife. The situation in human is not known. Some NTM are increasingly considered to be the causative organisms in clinical diseases in human. Such as *M. intracellulare* and *M. Kansasii*, and the present study has isolated too. They could be circulating at the human animal interface. Due to rise in immunesuppression, behavioural humans drinking untreated water and living in contact with cattle or close proximity with wild animals may be at risk of NTM infection. Due to fear of facing the global post antibiotic era, NTM detection and intervention is very important. Moreover, we have been observing the new paradigm of biofilm forming bacteria. Several NTM also have the ability to form biofilm. It is also important to know the prevalence and probably establish the status of biofilm formation which could be the other reason to increasingly multi drug resistance. The author highly recommends reciprocating the study on the human side to ascertain the status, too. Diagnosis of NTM requires specialized laboratory methods. Most of our local laboratories lack the facilities. It may be feasible to join proper diagnosis effort by other specialized laboratories in the country or outside. The direct sequencing could be adapted to routine work of clinical laboratories for accurate identification of NTM to the species level.

Acknowledgments

GERAFT Project is gratefully acknowledged for

their financial support of the study, and special thanks are also due to the Ministry of Livestock Development and Fisheries and TANAPA for granting authors this opportunity to pursue these studies.

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