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Excretion of *Mycobacterium tuberculosis* complex organisms or non-tuberculous *Mycobacteria* from tuberculin skin test positive cattle and buffaloes

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Abstract
A study was conducted to assess the respiratory excretion pattern of *Mycobacterium* species organisms from tuberculin skin test reactor cattle and buffaloes. Nasal secretions (n=33), milk samples (n=10) and prescapular lymph node aspirates (PSLA) (n=20) were collected from reactor animals and inoculated in BACTEC™ *Mycobacteria* growth indicator tubes (MGIT™) culture system. Ziehl Neelsen (ZN) staining of BACTEC positive samples revealed presence of acid fast organisms in nasal secretions (24.2%), milk samples (20%) and PSLA (15%). Polymerase chain reaction (PCR) for IS6110 gene revealed that no sample was positive for the presence of genome of *Mycobacterium tuberculosis* complex (MTBC) organisms. PCR with 16S rRNA detected presence of non-tuberculosis *Mycobacteria* (NTM) in 21.2% of nasal secretions, 20% of milk samples and 15% of PSLA collected. The earlier and rapid growth of NTM in the culture probably overwhelmed the growth of MTBC, which was confirmed in *in-vitro* growth curve experiments. Thus, the presence of NTM in the nasal secretions and other samples may mask the growth of MTBC; hence it could not be detected in the samples collected. This study concluded that the nasal swabbing may not be an ideal method for isolation of MTBC organisms from reactor animals wherein NTMs are abundantly present and post mortem tissue samples may be the appropriate source for MTBC isolation, in such samples.

Keywords: Non-tuberculous *mycobacteria*, BACTEC, *Mycobacterium tuberculosis* complex organisms, PCR-IS6110 and PCR-16S rRNA

Introduction
Non-tuberculous *Mycobacteria* (NTM) include all *Mycobacteria* other than the species belonging to the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae*. These are saprophytic environmental organisms and some of the NTM are now known to become opportunistic pathogen and cause disease in animals and humans (Kankya *et al*., 2011) [1]. NTM species causes severe respiratory infection and *M. avium*, *M. kansasii* and *M. abscessus* are few of the organisms involved in lung disease in humans (Johnson and Odell, 2014) [2]. The prevalence of NTM species is high in farm animals and wildlife and can cause granulomatous inflammation in the lymph nodes of cattle and other animals. The study was undertaken to assess the excretion pattern of *Mycobacterium* species from nasal secretion, milk and lymph node aspirates of tuberculous skin test (TST) positive cattle and buffaloes by using isolation of organisms in BACTEC™ MGIT™ cultures and identification using Ziehl-Neelsen (ZN) staining and polymerase chain reaction (PCR) for IS6110 and 16S rRNA.

Methodology
Sample collection
Nasal secretions, milk samples and lymph node aspirations were collected from TST reactor cattle/buffaloes to identify the excretion patterns of MTBC or NTM organisms. Nasal secretions were collected using tampon swab from 33 reactor cattle for 5 consecutive weeks. Milk samples were collected from 6 reactor cattle and 4 reactor buffaloes 2 times a day for 5 consecutive days. Lymph node aspirates were collected from prescapular lymph nodes of 20 reactor cattle at one time point only.

Culture and identification
These samples were decontaminated with 4% NaOH and inoculated in liquid media,

Mycobacterial growth indicator tube (MGIT) (Becton Dickson, USA). The MGIT tubes were incubated in the BACTEC MGIT 960 machine and growth monitored. The presence of *Mycobacterial* organisms in the nasal secretion, milk and prescapular lymph node aspirate (PSLA) were assessed by detection of growth units (GU) in BACTEC, demonstration of acid fast bacilli in BACTEC MGIT positive samples by ZN staining by conventional methods and detection of DNA by PCR for IS6110 for MTBC and PCR 16S rRNA for NTM.

Polymerase chain reaction

PCR assay was performed for the amplification of the 976 bp fragment of 16S rRNA gene to identify the presence of NTM in MGIT cultures 2 weeks post inoculation. The reaction was subjected to initial denaturation 95 ˚C for 10 minutes followed by 30 cycles of denaturation at 95 ˚C for 1 minute, annealing at 62 ˚C for 1 minute, extension at 72 ˚C for 1 minute and final extension at 72 ˚C for 5 minutes. PCR amplification was carried out in 15 µl reaction mixtures containing 1 µl of DNA template, 20 pM of 2 µl each of forward and reverse primers, 7.5 µl of GoTaq® Green Master Mix (Promega) and 2.5 µl of nuclease free water. The amplicons were analyzed by gel electrophoresis in a 1.7% agarose gel. The gels were visualized and documented using an automatic documentation system (Joao *et al*., 2014) [3].

The primers used were

16S rRNA F-AGAGTTTGATCMTGGCTCAG.

16S rRNA R-GTAAGGTTCTKCGCGTTGC.

Another PCR assay was performed for the amplification of the 445 bp fragment of IS6110 to identify the presence of MTBC. The annealing temperature was set at 58.9 ˚C and other conditions were similar to that for PCR for 16S rRNA. The following primers were used (Sweetline Anne *et al*., 2017) [4].

IS6110 F-GACCACGACCGAAGAATCCGCTG.

IS6110 R-CGGACAGGCCGAGTTTGGTCATC.

To compare the growth pattern of NTM and MTBC, known positive cultures of MTBC and NTM (each 3 nos.) were used. Equal volumes of MTBC and NTM cultures were inoculated in MGIT tubes and their growth monitored on a daily basis by comparing their growth units (GU) seen in the BACTEC machine. The mean (± SD) growth units were compared between MTBC and NTM organism on each day with the Student's '*t*' test ($p<0.05$).

Results

Tampon swabs were collected from 33 TST-positive cattle for 5 consecutive weeks and used in various assays for isolation and identification of MTBC/NTM. The positivity of the collected nasal tampon swabs in BACTEC culture, ZN staining, PCR for 16S rRNA and PCR for IS6110 are shown in Table 1. The excretion pattern of MTBC/NTM organism was highly erratic and inconsistent. No swabs were positive in BACTEC for more than 3 weeks consecutively. Only 4 of 33 animals (12.1%) had positive results in BACTEC isolation for 3 weeks. Only 9 (27.3%) animals had BACTEC positivity for 2 weeks. 10 (30.3%) animals were negative on all occasions and 10 (30.3%) animals were positive on one occasion only. Only 8 (24.2%) samples were positive for AFB staining, of which all samples had positive BACTEC culture at least on one occasion. None of the samples were positive for PCR IS6110 primer while 7 (21.2%) samples were positive for PCR 16S rRNA primers indicating growth of NTM over MTBC organism. Similar isolation results with milk samples and PSLA are shown in Table 2 and 3. Even with these samples PCR with IS6110 was negative while PCR 16S rRNA showed positivity in 2 milk samples (20%) and 3 PSLA samples (15%). The results of PCR for 16S rRNA are shown in Figure 1.

The growth curve of individual MTBC isolates and NTMs inoculated in MGIT is shown in Figure 2. The mean (±SD) GU of the MTBC and NTMs is shown in Table 4. NTM growth was seen from day 3 post-inoculation while in the case of MTBC it was seen only from day 6 post-inoculation. The growth of NTM was significantly higher ($p < 0.05$) from day 7 to 10 in MGIT as compared to the growth of MTBC.

Table 1: Nasal swabs collected from TST reactor cattle assayed by different tests

S. No.	Animal ID	BACTEC positivity					AFB	PCR-16S rRNA	PCR-IS6110
		1st week	2nd week	3rd week	4th week	5th week			
1	C1	-	Positive	-	-	-	-	-	-
2	C2	-	-	-	-	-	-	-	-
3	C3	-	-	-	-	-	-	-	-
4	C4	-	-	Positive	-	-	-	-	-
5	C5	-	-	-	Positive	-	-	-	-
6	C6	-	Positive	-	-	Positive	Positive	Positive	-
7	C7	Positive	Positive	Positive	-	-	Positive	Positive	-
8	C8	-	Positive	Positive	Positive	-	-	-	-
9	C9	-	-	-	-	-	-	-	-
10	C10	-	-	-	-	Positive	Positive	Positive	-
11	C11	Positive	-	-	-	-	-	-	-
12	C12	-	Positive	Positive	-	-	-	-	-
13	C13	-	Positive	-	-	-	-	-	-
14	C14	-	Positive	Positive	-	-	-	-	-
15	C15	-	-	-	-	-	-	-	-
16	C16	-	Positive	-	-	-	-	-	-
17	C17	-	-	-	-	-	-	-	-
18	C18	Positive	Positive	-	-	-	-	-	-
19	C19	Positive	-	-	-	-	-	-	-
20	C20	Positive	-	Positive	Positive	-	Positive	Positive	-
21	C21	Positive	-	-	Positive	Positive	Positive	Positive	-

22	C22	-	-	Positive	Positive	-	Positive	Positive	-
23	C23	-	Positive	-	-	-	-	-	-
24	C24	Positive	-	-	Positive	-	Positive	Positive	-
25	C25	Positive	-	Positive	-	-	Positive	-	-
26	C26	-	-	-	-	-	-	-	-
27	C27	-	-	-	-	-	-	-	-
28	C28	-	-	-	-	Positive	-	-	-
29	C29	-	-	-	-	-	-	-	-
30	C30	Positive	Positive	-	-	-	-	-	-
31	C31	-	-	Positive	-	-	-	-	-
32	C32		-	-	-	-	-	-	-
33	C33	-	-	-	-	-	-	-	-

Table 2: Milk samples collected from TST reactor cattle/buffalo assayed by different tests

S. No.	Animal ID	BACTEC positivity					AFB	PCR-16S rRNA	PCR-IS6110
		1st day	2nd day	3rd day	4th day	5th day			
1	C1	-	Positive	-	-	-	-	-	-
2	C2	-	-	-	-	-	-	-	-
3	C3	-	Positive	-	Positive	-	-	-	-
4	C4	-	-	-	-	-	-	-	-
5	C5	Positive	-	-	Positive	-	-	-	-
6	C6	-	Positive	Positive	-	-	Positive	Positive	-
7	B1	Positive	Positive	-	-	-	-	-	-
8	B2	-	-	-	-	-	-	-	-
9	B3	-	Positive	-	Positive	Positive	Positive	Positive	-
10	B4	-	-	-	-	-	-	-	-

Table 3: PSLA collected from TST reactor cattle assayed by different tests

S. No.	Animal ID	BACTEC positivity	AFB	PCR-16S rRNA	PCR-IS6110
2	C2	Positive	-	-	-
3	C3	-	-	-	-
4	C4	-	-	-	-
5	C5	Positive	-	-	-
6	C6	Positive	-	-	-
7	C7	-	-	-	-
8	C8	-	-	-	-
9	C9	-	-	-	-
10	C10	-	-	-	-
11	C11	Positive	-	-	-
12	C12	-	-	-	-
13	C13	Positive	-	-	-
14	C14	-	-	-	-
15	C15	-	-	-	-
16	C16	Positive	Positive	Positive	-
17	C17	-	-	-	-
18	C18	Positive	Positive	Positive	-
19	C19	Positive	Positive	Positive	-
20	C20	-	-	-	-

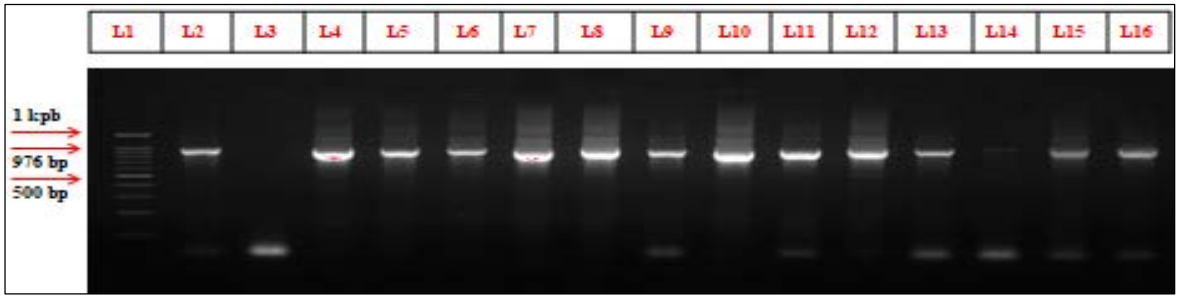

Fig 1: 16SrRNA-PCR of samples from lymph node, nasal swab and milk samples. The presence of an amplified product at 976 bp is considered positive

L1	DNA ladder 100bp	L9	NS1081 (positive)
L2	Positive control	L10	NS1064 (positive)
L3	Negative control	L11	NS1049 (positive)
L4	LN1143 (positive)	L12	NSJX25 (positive)
L5	LN1058 (positive)	L13	NS1037 (positive)
L6	LN1049 (positive)	L14	NS1109 (negative)
L7	NS1058 (positive)	L15	MLKJX27 (positive)
L8	NS1077 (positive)	L16	MLKM84 (positive)

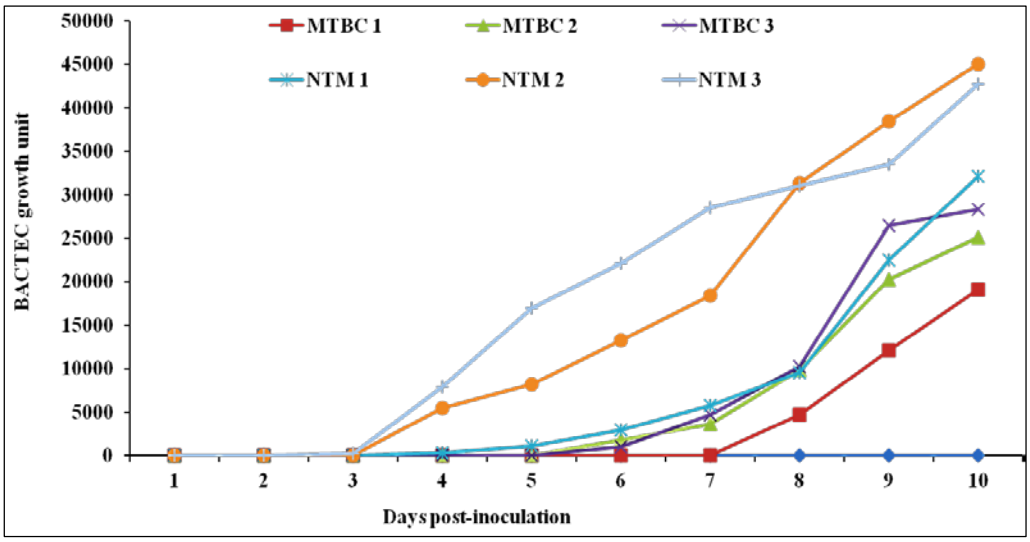

Fig 2: Growth curve of MTBC/NTM isolates in BACTEC

Table 4: Comparison of growth pattern of MTBC and NTM isolates

Days post inoculation	Mean ± SD of BACTEC growth units	
	MTBC (n=3)	NTM (n=3)
1	0	0
2	0	0
3	0	139 ± 99
4	0	4578 ± 3894
5	0	8780 ± 7943
6	927 ± 889	12755 ± 9594
7	2773 ± 2456	17555 ±11360*
8	8258 ± 3077	25315 ± 10203*
9	19629 ± 7189	32760 ± 6016*
10	24184 ± 4689	39935 ± 6900*

*indicates significant differences with MTBC growth units on that particular day (Student's *t*-test, $p < 0.05$)

Discussion

It is well known that bovine tuberculosis (bTB) spreads through aerosol route between animals in close proximity to each other. The TST is commonly done to identify the animals as positive or as reactors to bTB using purified protein derivative-bovine (PPD-B). It is clearly known that bTB is a herd disease and introduction of a bTB reactor/ positive animal could make entire herd infected within a short span of time. In our country, the test and slaughter policy is not feasible and it is a great challenge to decide what to do with a TST positive animal. Under this backdrop, it was decided to study the excretion pattern of TST-positive animals so that if the animals are actually found excreting the organisms such animals could be separated from the herd on a priority basis. Knowing that the excretion pattern in positive animals could be intermittent and inconsistent, the TST animals were swabbed for 5 consecutive weeks and used for isolation in BACTEC. Any growth observed in MGIT was used in ZN staining followed by PCR to detect MTBC (IS6110) and/or NTM (16S rRNA).

The gold standard method for diagnosis of tuberculosis in humans uses a similar strategy. Sputum collected from tuberculosis-suspected individuals is inoculated in MGIT and positive cultures are confirmed as belonging to MTBC using immune chromatographic strip test. The strip test detects *Mycobacterium tuberculosis* specific early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and MPB64 present in the positive culture. Any sample, although showed evidence of growth in MGIT but no positive result in the strip test is considered negative for TB since it does not involve MTBC organisms.

In this study it was hoped to isolate organisms belongs to MTBC from TST positive animals at least on few occasions of nasal swabbing. However, although growth in MGIT was observed in many cases and smears made from these cultures showed the presence of acid fast organisms, all the samples were negative for IS6110 PCR indicating that they do not belong to MTBC organisms. Hence these samples were used

in 16S rRNA PCR to rule out the presence of NTM and it was found that all the samples were positive for NTM. This indicated that under conditions of nasal swabbing in bovine, attempts to isolate MTBC may be masked by the fast growth of NTM in these samples. To confirm this, the growth pattern of NTM and MTBC were compared, wherein it was found that the growth of NTM overwhelms the growth of MTBC.

Non tuberculous *Mycobacteria* are saprophytic organisms likely to be found in environment and also in the nasal cavity of bovines. Our study detected the presence of NTM organisms in nasal secretions, milk samples and PSLA samples collected from TST positive cattle and buffaloes. This is also fortified for the fact that the skin reactivity to PPD-avian (PPD-A) in these animals were also high (data not shown). Most of the NTMs identified in animal tissues are found in the environment. Gcebe *et al*. (2013) [5] recovered 16 known NTM species from cattle and buffaloes by using nasal and pharyngeal swabs and characterized by sequencing a partial region of 16Sr RNA. Kankya *et al*. (2011) [1] isolated NTM species from animal faecal samples. Milk may be considered one of the potential sources for NTM in human infection. Sgarioni *et al*. (2014) [6] detected NTMs in raw and pasteurized milk; no *M. bovis* or other MTBC was detected.

Malama *et al*. (2014) [7] reported that they have isolated NTMs from tuberculosis-like lesions found in lymph node tissues of cattle. Gcebe *et al*., (2017) [8] observed tuberculosis like lesions and isolated NTM species from lung, heart, and mesenteric and bronchial lymph nodes of wild animals. They could not recover any MTBC organisms from theses lesions. This implies the pathogenic potential of NTM in animals. Hernandez-Jarguin *et al*., (2020) [9] observed granulomatous and pyogranulomatous lesions in lymph nodes of carcasses of cattle and isolated NTMs.

Thus it is reasonable to conclude that isolation using nasal swab may not be ideal method for the isolation of MTBC especially in environment where NTMs are abundantly present. The only alternate method available is trying isolation from bTB-suspected lesions on post mortem examination. Such an approach could yield positive cultures of MTBC organism from animals that died, which previously showed negative during MTBC isolation by nasal swabbing. Alternatively molecular methods such as PCR and real-time PCR may be applied directly on the swabs and the isolation may be attempted only on samples where the genome of MTBC and no NTM has been detected. The importance of NTM is on the rise in human cases causing several pulmonary and extra pulmonary lesions.

Conclusion

This study concluded that the presence of NTM in the samples collected from the animals may mask the growth of MTBC organisms. The abundant and earlier growth of NTM is likely to overwhelm the growth of MTBC which could not be detected in the samples collected from TST positive cattle and buffaloes. Hence, isolation of MTBC organisms by nasal swabbing may not be an ideal method wherein NTMs are abundantly present in the environment. Isolation from tuberculous lesions during post mortem examination would be the preferred strategy for isolation for genomic characterization.

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