Int.J.Curr.Microbiol.App.Sci (2019) *8*(2): 1197-1205

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 8 Number 02 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.802.139

Surveillance and Species Identification of Mycobacteria in Cattle from Abattoirs of Assam and Meghalaya

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Keywords

AFB, IFN-γ, *Mycobacterium bovis*, Necropsy, PCR

Article Info

Accepted:
10 January 2019
Available Online:
10 February 2019

ABSTRACT

The study was carried out to investigate bovine tuberculosis and diagnostic potential of IFN-γ assay and necropsy inspection in different abattoirs of Assam and Meghalaya, including its species identification. A total number of 234 animals were screened by IFN-γ and necropsy inspection. IFN-γ was performed according to kit procedures and carcasses were inspected for any gross visible lesion. Species identification was confirmed by biochemical test (viz. Nitrate reduction test, Pyrazinamidase test and Niacin detection test) and PCR targeting *pncA* and *oxyR* gene. The inter-rater agreement (weighted kappa) among different screening tests was analysed using standard software. Gross visual lesions were found in 48 (20.51%) carcasses whereas 43 (18.38%) animals were reactive to IFN-γ. Suspected 119 tissue samples were collected from 48 animals. Pre-culture stain revealed 83 (69.75%) and culture growths were from 96 (80.67%) tissue samples. Highest lesions were recorded in lymph nodes (56.30%) followed by lungs (16.80%) and liver (8.40%). Pre-scapular (38.80%) and retropharyngeal (26.86%) contribute more lesions than other lymph nodes. The sensitivity and specificity of IFN-γ was 81.58 % and 98.29% respectively. Very good Inter-rater agreements (kappa) were observed between IFN-γ, culture and pre-culture stains and good agreement between IFN-γ and necropsy inspection. Our results indicate visual inspection may serve as good screening method for tuberculosis infected carcasses although IFN-γ assay before slaughter may give brief idea about the infection. The study confirms the endemic status of bovine tuberculosis in these areas of north east India.

Introduction

In many developing countries bovine tuberculosis (BTB) is a major infectious disease among domesticated animals and certain captive wild animals. It is estimated that *M. bovis* is responsible for about 5% of all TB infection in human (Cosivi *et al.,* 1998 and Michel *et al.,* 2010). Farmers, slaughterhouse workers, animal keepers at zoo as well as veterinary professionals are at high risk to the exposure of BTB infection

through contact with infected livestock or their carcasses (Elmonir and Ramadan, 2016). India possesses more than 16% of world cattle population. Cattles are also considered as natural host of *M. bovis*. Milk from healthy lactating cows had been reported to shed *M. bovis* bacilli (Danbirni *et al.,* 2010). Ingestion of beef from infected cattle can be a major threat to public health as cooking may not always be an effective against *M. bovis* infection (van der Merwe *et al.,* 2009). In the case of dairy milk however, pasteurisation minimizes the risk of infection. In *M. bovis* infected cattle, CD4 T-cells produces IFN-γ leading to the activation of macrophage, with CD8 T-cells greater involvement in the lysis of infected cells (Skinner *et al.,* 2003). Okafor *et al.,* (2013) documented that IFN-γ response is sufficient to classify cattle as positive for tuberculosis. Polymerase chain reaction (PCR) of *pncA* and *oxyR* gene was evaluated for species specification for *M. bovis* and *M. tuberculosis*. As *M. bovis* is resistant to pyrazinamide, species identification will also help for treatment of individuals.

Abattoirs, butcher shops provide an ideal environment as a monitoring point for the screening of carcasses for BTB. Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection of cattle, but infection may be occurred by contaminated material (Barua *et al.,* 2016). Characteristic tuberculous lesions occur most frequently in the lungs, liver and lymph nodes.

The present study was undertaken for the purpose to investigate the infection of BTB in abattoirs, butcher shop and meat market in some parts of Assam and nearby state of Meghalaya. In this study, we also investigated the efficiency of IFN-γ assay and necropsy findings based on bacterial culture, biochemical tests and polymerase chain reaction (PCR) for species identification.

Materials and Methods

Study site and cattle breeds

The current study was carried out in different abattoirs, butcher shop and meat market (beef) located in various places of Assam and nearby state Meghalaya. The slaughter environment is mostly unhygienic and unorganized. In Assam cattle are reared mostly for milk and livelihood. Predominantly local indigenous constitutes about 60 % and others are jersey crossbred in both the states.

IFN-γ assay

Blood samples were collected aseptically before slaughter for IFN-γ assay. It was performed according to kit procedures (RayBio bovine IFN-gamma ELISA kit). Briefly, bovine IFN-γ was used as a standard at 30 ng/ml, 12 ng/ml, 4.80 ng/ml, 1.92 ng/ml, 0.768 ng/ml, 0.307ng/ml, 0.123 ng/ml, along with the positive and negative controls (RPMI 1640). Samples were read at a wavelength of 450 nm to calculate optical density. A sample was considered as positive when the difference between mean optical density value of a negative control with mean optical density value of sample is equal or higher than 0.100.

Gross necropsy

All the carcasses were inspected for any gross visible lesion suspected of tuberculosis. Organs and tissue samples were collected from all the carcasses for further analysis. In this study, an animal was considered positive on necropsy if 1 or more lymph nodes or other tissues contained focal or multifocal abscesses or granulomas. Although some samples with no visible lesions were also processed further for tests.

Pre-culture staining (PCS)

Ziehl-Neelson (ZN) staining for the detection of acid-fast bacteria (AFB) was performed on all tissue samples. A sample was considered positive for tuberculosis if there was evidence of granulomatous inflammation associated with focal necrosis or mineralization and/or if there was identification of AFB on the ZN stain.

Mycobacterial culture and species identification

Fresh and stored tissue samples were macerated and decontaminated using NALC and inoculated on to Lowenstein Jensen (LJ) media. Briefly, approximately 1g of tissue exhibiting gross visible lesions was sliced and homogenized and then subjected for decontamination.

The supernatant was discarded and the pellet formed re-suspended in 300µl of phosphate buffered saline (140mM NaCl, 26mM KCl, 10.0mM Na_2HPO_4 and 1.7mM KH_2PO_4). Then the re-suspended pellets were inoculated in duplicates onto LJ slants (one incorporating glycerol and the other pyruvate). LJ slants were incubated at 37°C and observed weekly for eight weeks. Using a sterile 0.1 µl plastic loop, the re-suspended pellets were spread and fixed at 80°C (for 10 min) onto a labelled slide. The slides were subjected for staining with modified ZN stain.

Biochemical analysis were performed for species identification of mycobacteria as per standard protocol, such as Nitrate reduction test (Kubica and Wayne, 1984), Pyrazinamidase test (Wayne, 1974) and Niacin detection test (Gadre*et. al*., 1995). DNA was isolated from bacterial culture and PCR was done targeting *pncA* and *oxyR* gene as per De Los Monteros *et al.,* (1998).

Statistical analysis

Data analysis was carried out in Microsoft excel version 2010. Sensitivity and specificity were calculated as per Bassessar*et al.,* (2014). The inter-rater agreement (weighted kappa) among different screening tests were analysed using MedCalc Statistical Software (trial version 15.8 MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2015). Kappa values were interpreted according to Altman (1991).

Results and Discussion

In the current study, we assayed IFN-γ in animals before slaughter and necropsy tissue samples with lesions suggestive of mycobacterial infection from abattoirs, butcher shop and meat market using ZN microscopy and compared the results with those of culture, biochemical tests and PCR.

A total of 234 animals were pooled from slaughter house and butcher shop based on their debilitating health condition. Only 43 (18.38%) showed reactive to IFN-γ (Table 1). The sensitivity and specificity of IFN-γ was 81.58 % and 98.29% respectively (Table 2), which is agreeable with Gormley *et al.,* (2013) where sensitivity of IFN-γ varied between 73.0 -100% and specificity with a range of 85.0–99.6%.

However, because a few of the animal each had more than one organ presenting lesions, 119 samples of suspicious organs were obtained from 48 animals. In terms of organ involvement, the majority lesions were found in lymph nodes (67, 56.30 %) followed by lungs (20, 16.80%) and liver (10, 8.40%) respectively (Table 3), which is comparable with Teklu *et al.,* (2004) and Stefan *et al.,* (2009). Out of 67 lymph nodes, prescapular and retro pharyngeal lymph nodes contribute more (Table 4). PCS revealed that, out of the

48 carcasses disclosing suspicious lesions at necropsy, 70.83% (34/48) furnished lesions samples tested positive for AFB contributing 69.75% of total suspected samples. In terms of the 119 individual organ sample analysed, lymph nodes represented the highest number of pre-culture stain positive samples, 42.85% (51/119), followed by lung tissues, 15.12% (18/119) and then liver 6.72% (8/119). For the 67 lymph nodes screened in PCS, prescapular lymph nodes showed 35.82% (24/67) positive for AFB followed by retro pharyngeal 20.89% (14/67) (Table 4).

In terms of overall organ distribution (n = 119), the number of lesions in lymph nodes was higher than lungs and liver but the fractions of ZN positive samples out of each organ category was different, *e.g.* in lung tissue (90.0 % or 18/20) and liver (80.0% or 8/10) were higher than that in lymph nodes (76.12% or 51/67). Low ZN-positive results in the lymph nodes in this study may be due to the low rate of survival of mycobacteria in central caseation of lymph node (Cassidy, 2006) or instability of bacterial structure as a result of some immune reactions that occur in response to infection by mycobacteria (Guitierrez *et al.,* 1993)

All the suspicious 119 samples were processed and inoculated onto LJ slants, 80.67% (96/119) grew successfully, 5.2% (n = 3/119) were contaminated and 16.81% (20/119) did not show any growths. In terms of cultured tissue distribution, out of the 96 successful culture isolates obtained, 61 (or 51.26%) were from lymph nodes. This finding was in consistent with many studies (Aylate *et al.,* 2013; Barua *et al.,* 2016; Shitaye *et al.,* 2006; Youssef and Ahmed, 2014).

Table.1 Results of different screening methods for detection of mycobacteria infection in 234 animals

	Total	Positive	Negative
Visual lesions (suspected)	234	48 (20.51%)	186 (79.48%)
pre culture stain		37 (15.81%)	197 (84.19%)
Culture		39 (16.67%)	195 (83.33%)
IFN-γ		43 (18.38%)	191 (81.62%)

Table.2 Sensitivity and specificity of carcass inspection (necropsy), pre culture stain (PCS) and IFN-γ based on culture and PCR as gold standard

Parameters	No. of animal	Sensitivity	Specificity
True Positive	31		
True Negative	173		
IFN-γ false Positive	7	81.58%	98.29%
IFN-γ False Negative	3		
PCS false positive	3	91.18%	97.19%
PCS false negative	5		
necropsy false positive	17	64.58%	95.58%
necropsy false negative	8		

Table.3 Distribution of various organs involved in mycobacteria infection. (n = 119)

Organs	Suspected lesions	PCS +ve	culture +ve
Lung	20 (16.80%)	18 (15.12%)	19 (15.97%)
Lymph node	67 (56.30%)	51 (42.85%)	61 (51.26%)
Liver	10 (8.40%)	8 (6.72%)	10 (8.40%)
Spleen	7 (5.88%)	2 (1.68%)	2 (1.68%)
Pleura	6 (5.04%)	2 (1.68%)	2 (1.68%)
Peritoneum	5 (4.20%)	1 (0.84%)	1 (0.84%)
Uterus	4 (3.36%)	1 (0.84%)	1 (0.84%)
Total	119	83 (69.75%)	96 (80.67%)

Table.4 Distribution of different lymph nodes involved in mycobacteria infection

(n = 67)

lymph nodes	lesion +ve	pcs +ve	culture +ve
Bronchial LN	6 (8.95%)	5 (7.46%)	6 (8.95%)
Messenteric LN	9 (13.43%)	5 (7.46%)	8 (11.94%)
Prescapular LN	26 (38.80%)	24 (35.82%)	26 (38.80%)
Retro Pharyngeal LN	18 (26.86%)	14 (20.89%)	18 (26.86%)
Supra mammary LN	5 (7.46%)	2 (2.98%)	2 (2.98%)
Mediastinal LN	3 (4.48%)	1 (1.49%)	1 (1.49%)
Total	67	51 (76.12%)	61 (91.04%)

Table.5 Inter rater agreement (kappa) between different screening methods

	PCR +ve	PCR -ve	kappa	IFN-γ +ve	IFN-γ -ve	kappa	PCS +ve	PCS -ve	kappa
Lesion positive	31	17	0.648	33	15	0.659	34	14	0.757
Lesion negative	8	178		10	176		3	183	
PCS positive	34	3	0.874	35	2	0.849			
PCS negative	5	192		8	189				
IFN-γ positive	36	7	0.852						
IFN-γ negative	3	188							

Fig.1 Gross visible lesion of a) lung b) liver c) lymph node d) spleen e) peritoneum f) uterus

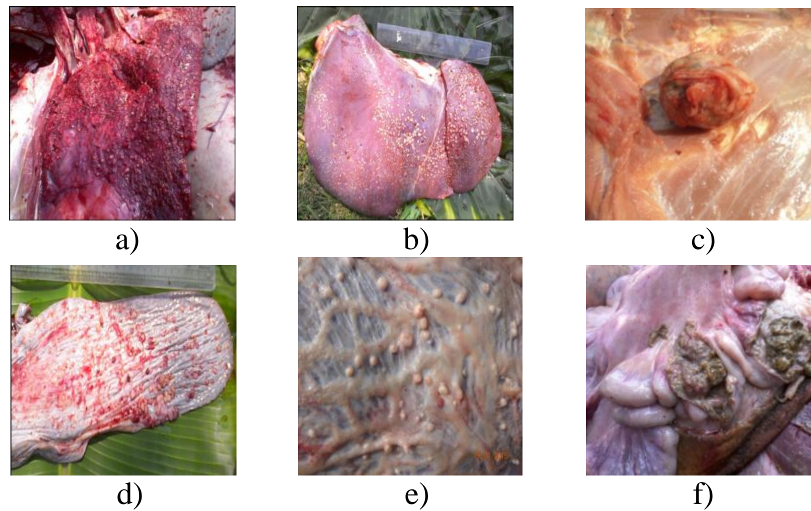

Fig. 2 Agarose gel electrophoresis showing presence of a) *pnc*A (185bp) and b) *oxyR* (280bp) gene in *M. bovis* (L1 and L3) but absent in *M. tuberculosis* (L2 and L4). Lane M indicates 100bp marker

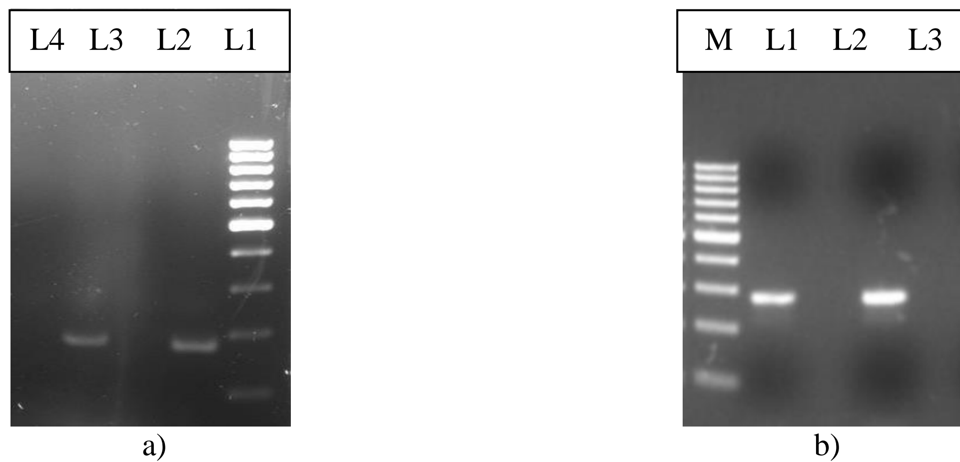

Fig.3 Unhygienic and unorganized cattle slaughter environment

Out of the 234 carcasses, 37 showed positive by pre-culture stain of which in 3 animals no visible lesions observed. This may indicate that ZN pre culture microscopy is quite good at correctly identifying samples.

All the culture positive samples showed negative for nitrate, pyrazinamidase, niacin test which indicates positive for *M. bovis*. In PCR, all the culture samples were found to be positive for BTB, showing band at 185bp of *pnc*A and 280bp of *oxyR* gene (Fig. 2) specific for *M. bovis*, which is in agreement with Barua*et al.,* (2017).

Moreover, in the present study the inter ratter agreement (kappa) between PCR and PCS (0.874), IFN-γ and PCS (0.849), PCR and IFN-γ (0.852) were found to be very good. Necropsy analysis showed a good agreement (0.60-0.80) with PCR, PCS and IFN-γ (Table 5).

Acknowledgement

Authors are thankful to ICAR, New Delhi for funding the "Outreach Project on Zoonotic diseases" and Director of Research (Veterinary) for necessary facilities to carry out the research. Due acknowledgement is also extended to the abattoir workers for providing samples.

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How to cite this article:

Acheenta G. Barua, Koushik Kakoty, Pranjal M. Nath, Himangshu Raj, JyotiPawan Chutia and Pranab Koch. 2019. Surveillance and Species Identification of Mycobacteria in Cattle from Abattoirs of Assam and Meghalaya. *Int.J.Curr.Microbiol.App.Sci.* 8(02): 1197-1205.
doi: https://doi.org/10.20546/ijcmas.2019.802.139