Title: Zoonotic tuberculosis of the central nervous system: diagnostic challenges and prospects

Authors: Prachi R. Bapat [1], Seema D. Shekhawat [1], AliabbasA.Husain [1], Renuka S. Dodkey [1], Hatim F. Daginawala [1], Lokendra K. Singh [1], Rajpal S. Kashyap [1]*

[1] Research Centre, Central India Institute of Medical Sciences, Nagpur, Maharashtra, India.

To appear in: Basic and Clinical Neuroscience

Received date: 29.11.2016

Accepted date: 11.01.2017

Please cite this article as:

Bapat, P. R., Shekhawat, S. D., Husain, A. A., Renuka, S., Dodkey, R. S., Daginawala H. F., et al. (In Press). Zoonotic tuberculosis of the central nervous system: diagnostic challenges and prospects. *Basic and Clinical Neuroscience.* Just Accepted publication Jan. 11, 2018. doi:

Zoonotic tuberculosis of the central nervous system: diagnostic challenges and prospects

Prachi R. Bapat [a], Seema D. Shekhawat [a], AliabbasA.Husain [a], Renuka S. Dodkey [a], Hatim F. Daginawala [a], Lokendra K. Singh, Rajpal S. Kashyap [a]*

[a]Research Centre, Central India Institute of Medical Sciences, Nagpur, Maharashtra, India.

Number of text pages (whole manuscript): 21

Number of Figures: 4

Number of Tables: 6

Running Title: Implications of Zoonotic TB on CNS

Corresponding Author

Dr. Rajpal S. Kashyap*

Address: Biochemistry Research Laboratory, Central India Institute of Medical Sciences, 88/2, Bajaj Nagar, Nagpur, Maharashtra, India.

Telephone: +91-712-2233381/2236441

Fax: 0712-2236416

E-mail: raj_ciims@rediffmail.com

Abstract

Diagnosis of tuberculous meningitis (TBM) has remained a challenge due to its insidious onset and failure of conventional diagnostic tests. The present study was undertaken to accurately identify the mycobacterial pathogen in the CSF of TBM patients with poor prognosis. We retrospectively recruited 224 TBM and 34 Non-TBM patients admitted to Central India Institute of Medical Sciences, Nagpur in the year 2014. The CSF samples of these patients were subjected to a duplex PCR assay for species specific identification of the causative pathogen. *M.bovis* was detected in 7% (18) of the samples and infection with *M.tuberculosis* was detected in 32.9% (85). 14% (36) of the samples were culture positive; however the mycobacterial pathogens could not be differentiated to the species level. This study emphasizes the potentially vital importance of *M.bovis* identification for appropriate patient management and demonstrates the persistent significance of *M.bovis* as a zoonotic pathogen.

Keywords: Duplex PCR, *Mycobacterium bovis*, Tuberculous meningitis, Zoonoses

1. Introduction

The last decade has witnessed shifting trends in tuberculosis (TB) infection, with extra pulmonary tuberculosis (EPTB) emerging as an important entity (Jain, 2011). Tuberculosis of the central nervous system (CNS) is the most fatal extra-pulmonary disease that affects nearly 10% of the population globally affected with TB. The estimated mortality due to tuberculous meningitis (TBM) in India is 1.5 per 100,000 population (Murthy, 2010; Kaur, et al., 2015). Diagnosis of TBM has remained a challenge to the clinician due to nonspecific clinical manifestation which varies widely thus creating major obstacle in initiation of treatment. Moreover, limited sensitivity of conventional diagnostic tests (smear microscopy and culture) due to paucilbacillary nature of samples also contributes to delayed diagnosis (Jain, 2011; Purohit & Mustafa, 2015).

Different studies have reported the possibility of association between variables such as age, stage of disease, clinical characteristics, mycobacterial isolation from cerebrospinal fluid (CSF) etc and the manifestation of TBM. The reason for poor prognosis has been ascertained to the lack of appropriate diagnostic tools for the identification of the causative organism in some reports (Kaur, et al., 2015; Ahmadinejad, et al., 2002). Despite being a TB endemic country, limited efforts have been made towards preventive measures to improve the subsequent outcome in TBM patients. Despite the serious consequences following mycobacterial infection of the CNS, our understanding of the neuro- and immunopathogenesis of cerebral mycobacterial infection is limited.

Our laboratory, for the past decade, has been working on the development of immunodiagnostic and molecular tools for clinical evaluation and appropriate management of TBM patients admitted to Central India Institute of Medical Sciences (CIIMS), a tertiary healthcare facility providing medical services to the Central Indian population (Kashyap, et al., 2006; Kashyap, et al., 2007;

Deshpande, et al., 2008). Majority (70%) of those admitted here, are neurology and neurosurgery patients.

In our experience, a considerable number of TBM patients after receiving the standard drug regimen do not respond to the treatment and show neurological sequelae. The present work was thus planned as a retrospective study to assess the predictors of mortality or poor prognosis in patients diagnosed with TBM.

2. Materials and Methods

2.1 Ethics Statement

The study was approved by Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur and is in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written consents were taken from each participant after detailed oral explanation about the study.

2.2 Study Design and Participants

We retrospectively reviewed the medical files of all patients with the diagnosis of tubercular meningitis (TBM) admitted in the year 2014 to Central India Institute of Medical Sciences (CIIMS). From each medical case file, the patient's history, physical findings, chest radiographs and reports of laboratory investigations were assessed to obtain the necessary information about diagnosis of TBM. For each patient, demographic information (age, gender), and clinical characteristics were also recorded.

Diagnosis of TBM was done according to internationally recognized clinical, radiological and laboratory criteria (Torok, 2015). Clinical criteria included fever, headache and neck stiffness.

Laboratory criteria included CSF pleocytosis greater than 10 cells per mm^3 or proteins above 30 mg per deciliter. Radiological criteria included hydrocephalus, tuberculomas, cerebral infarcts, meningeal enhancements or exudates. In addition, the results of culture or staining of clinical sample, polymerase chain reaction (PCR) test and Ag/Ab test in the CSF, tuberculin skin test were collected. A definite diagnosis of meningeal tuberculosis was done when a positive culture or stain was present. A probable diagnosis was done when the clinical picture was compatible. A possible diagnosis was done when there was a suggestive clinical picture, and improvement with anti-tuberculosis treatment.

Two hundred seventy eight cases were diagnosed with TBM in the year 2014. Cases with incomplete data or uncertain diagnosis were excluded from the study. Based on the above mentioned criteria, we separated two hundred twenty four cases and followed them for a duration of 6 months and 12 months until 2015. These cases were clinically evaluated for new neurological complications including new formation or enlargement of cerebral lesions, development of hydrocephalus, and new onset of seizures or cranial nerve paralysis, occurrence of intracranial hypertension, coma, and death or significant signs of improvement with no new neurological deficits or symptoms.

Of the two hundred twenty four TBM cases, forty four cases emigrated or were lost to follow-up within the first six months of diagnosis. Of the remaining one hundred eighty patients, 53.9% (97) cases recovered completely, 36.1% (65) showed neurological deterioration, and 10% (18) died. After 12 months follow-up period, these rates changed to 82.3% (84) recovered cases, 15.7% (16) deteriorated cases and 2% (2) expired cases. The study work flow is represented in Figure 1. Along with the TBM group, a control group was also included in the study consisting of 34 Non-TBM

patients with viral (15) and fungal (4) meningitis and patients of non-infectious illnesses of the central nervous system (15).

2.3 Species specific identification of the pathogenic organism

The potential for detecting mixed/new pathogenic infections in patients with poor prognosis led to the formulation of a new strategy for differential diagnosis against the pathogenic mycobacteria. For this purpose, Cerebrospinal fluid (CSF) samples of TBM and non-TBM patients were processed for accurate identification of mycobacterial species using a duplex PCR assay targeting the regions of difference (RD) 1 and 4 in a single reaction.

2.3.1 Sample collection

CSF samples were collected under aseptic conditions by standard lumbar puncture. Five hundred microliter to 1 ml of sample was available for the study. Samples were stored at -20°C, prior to processing for target DNA for duplex PCR and culturing in the BACT alert system.

2.3.2 DNA isolation and quantification

DNA was extracted from CSF samples by modification of the phenol chloroform extraction method described by Deshpande, et al. (2007); wherein 500 µl of sample was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 500 µl of PBS, 15 µl 10% SDS and 3 µl proteinase K (20 mg/ml), mixed and incubated at 55°C for one and a half hour. After incubation, 100 µl of 5 M NaCl and 80 µl of high-salt CTAB buffer (containing 4 M NaCl, 1.8% CTAB (cetyl-trimethyl-ammonium bromide) was added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (350 µl) of phenol and of chloroform-isoamyl

alcohol (24:1) was added, mixed thoroughly and centrifuged for 10 min in a microcentrifuge at 12,000 r.p.m. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform-isoamyl alcohol (1:1) was added followed by a 10 min spin at 12,000 r.p.m. The aqueous layer was separated and then mixed with 30 µl of 3M sodium acetate and 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 70% ethanol, dried and re-suspended in 30 µl of Tris-EDTA (TE) buffer and were stored at -20°C before use. DNA concentrations for all samples and strains used in this study were determined with the Quant-iTdsDNA HS assay kit using a Qubit fluorometer (Invitrogen).

2.3.3 Duplex PCR

For determination to the species level of the mycobacterial pathogens, namely, Mycobacterium tuberculosis (*M.tb),* Mycobacterium bovis (*M. bovis)* and *M. bovis* Bacilli Calmette Guérin (BCG), two genetic regions RD4 and RD1 were amplified using a duplex approach. Primers used in this study are shown in Table 1.

RD4 is a region of difference in the bovine lineage. The use of RD4 flanking primers ensured that the PCR products were formed only if the deletion was present (Taylor, Worth, & Palmer, 2007). The genes of the RD1 region belong to the *esat6* gene cluster. ESAT-6 is a potent stimulator of the immune system, and is an antigen recognized during the early stages of infection. RD1 region of *M.tb* is considered to be the primary attenuating deletion in the related vaccine strain *M.bovis* BCG (Halse, Escuyer, & Musser, 2011). (Figure 2)

The duplex PCR reactions were carried out using 10X PCR buffer, 1.5 $MgCl_2$, 0.8 mM dNTPs, 0.4 µM of RD1F/R and 0.2 µM of RD4F/R and 1.25U of *Taq* DNA polymerase. The amplification procedure consisted of initial denaturation at 95°C for 7 min and 35 cycles each of denaturation at

95°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min followed by a final extension step at 72°C for 10 min.

2.3.4 PCR minimum detection limits

The sensitivity of the method was determined using serially diluted purified genomic DNA solutions-ten fold dilution from 10ng/µl to 1 fg/µl, extracted from *M.tb* (ATCC 25177), *M. bovis* (ATCC BAA-935) and *M. bovis* BCG Pasteur (ATCC 35734). For the specificity study, the concentration of the DNA solution from each reference strain was adjusted to10 ng/µl and used.

2.3.5 Interpretation of the Results by Duplex PCR

The PCR amplicons were analyzed on a 2% agarose gel and stained with ethidium bromide. The amplified products were then visualized under UV light. Comparative analysis on electrophoresis of the PCR products generated by the two sets of primer pairs showed the ability to distinguish between *M.tuberculosis, M.bovis* and *M.bovis* BCG. The duplex PCR was considered as positive for *M.bovis* when bands of 176-bp and 110-bp were seen; positive for *M. bovis* BCG when band of only 176-bp was present and positive for *M. tuberculosis* when band of only 110-bp was present.

2.3.6 DNA Sequencing Analysis

The PCR products were purified and sequenced by Sanger's dideoxy chain termination method at the SciGenom Labs, Cochin India. Sequences were verified by BLAST search using the NCBI website.

2.3.7 AFB Drug Susceptibility

The positive culture isolates were subjected to antibiotic sensitivity using five first line drugs viz; streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide (SIREP) at Metropolis Healthcare Limited, Mumbai. In-vitro drug susceptibility testing was performed by incorporation of required drug concentration and subsequent inoculation of modified Midlebrook 7H9 Broth with standardized inoculum and incubated at 35° C in the automated BACTEC MGIT 960 system. Strains were declared resistant if growth of more than 20 colonies were observed at drug concentrations as described by Rai, Bhattacharya and Kamal. (2007).

2.4 Statistical analysis

Demographic variables and clinical characteristics were compared between TBM and Non-TBM groups. The Chi square test for categorical variables was used to test for differences between the groups and p-values ≤ 0.05 were considered statistically significant. All tests were performed using MedCalc statistical software (version 10.1.2.0).

3. Results

3.1 Classification of recruited participants

Based on the clinical, radiological and CSF findings, the recruited participants were categorized into Definitive TBM (n=24), Probable TBM (n=123), Possible TBM (n=77) and Non-TBM cases (n=34) as represented in Table 2.

3.2 Clinical characteristics of recruited participants

The overall male to female ratio of recruited cases was 1.7 (164/ 94). For TBM patients, the male to female ratio was 1.5 (134/90) and 7.5 (30/4) for Non-TBM cases. The difference was statistically significant ($P < 0.05$). The median age of TBM patients (37.19 years) and that of Non-TBM cases (37.01 years) was nearly similar (Table 3). Significantly higher proportion of TBM cases had headache, fever, altered sensorium and neck stiffness as compared to Non-TBM cases ($P<0.01$). On the other hand, higher proportion of Non-TBM cases had hallucinations as compared to TBM cases. Higher proportion of TBM cases had seizures and altered consciousness as compared to Non-TBM cases however; not statistically significant. The mean duration of illness was also significantly higher for TBM cases as compared to Non-TBM cases ($P<0.0001$). The proportion of cases that showed hydrocephalus and meningeal enhancement was also higher in TBM cases compared to Non-TBM cases. Laboratory findings indicated that the mean levels of protein and cell count in the TBM cases were significantly higher than that in the Non-TBM cases ($P <0.05$). The glucose levels, on the other hand were significantly lower in the Non-TBM cases as compared to the TBM cases ($P < 0.0001$).

3.3 Follow-up analysis

Out of the total 224 TBM cases, 180 patients were followed up for 6 months and 102 patients could be followed upto 12 months. The radiological findings in the 6 month follow-up cases indicated that 22.8% had hydrocephalus, 3.9% had meningeal enhancements and 1 case suffered from stroke. In the second follow-up, 4.9% cases showed hydrocephalus, 12.7% had meningeal enhancement. Protein, glucose and cell count were found to be abnormal in 26.7%, 12.8% and 18.9 % cases during 6 months follow up. After 12 months follow-up, these rates changed to 17.6%, 14.7% and 12.7% for protein, glucose and total cell count respectively. Table 4 indicates the clinical and radiological findings of follow-up TBM cases.

3.4 Detection and differentiation of M.tuberculosis and M .bovis

The detection and differentiation of *M.tb* and *M .bovis* in CSF samples is represented in Figure 3. The PCR products in these samples were found to align with the RD4 and RD1 regions of *M.bovis* as represented in Figure 4A and 4B. Of the 258 samples, 103 samples (39.9%) were positive by the duplex PCR assay. *M.bovis* was detected in 7% (18) of the samples. Infection with *M.tb* was detected in 32.9% (85) of the samples. 14% of the samples were positive for Bactec culture, *M.tb* and/or *M.bovis* however could not be differentiated to the species level of the mycobacterial pathogens. The comparative efficiency of duplex PCR and Bactec culture for detection of mycobacteria has been represented in Table 5.

3.5 Drug susceptibility testing

In-vitro drug susceptibility testing to first line antitubercular drugs (SIREP) was carried out and the susceptibility pattern is shown in Table 6. One of the culture isolate was found to be resistant

to pyrazinamide at a critical concentration of 100 μg/ml. No other isolate showed resistance to the 1st line antitubercular drugs.

4. Discussion

The diagnosis of TBM has remained a challenge due to low efficiency and prolonged time taken by culture methods and conventional biochemical techniques. The paucibacillary nature of CSF has also been an additional impediment in the accurate diagnosis of the disease (Rock, Olin, & Baker CA, 2008; Marx & Chan, 2011).

We began this study with an aim to understand the variety of presentations of TBM including fatal and irrevocable effects occurring in significant number of cases. We selected carefully, those patients who were diagnosed with TBM, and followed them for a span of one year to assess their neurological condition. With an underlying objective to clinically evaluate TBM patients with poor prognosis, we developed an assay based on molecular detection of RD regions to accurately identify the causative organism in CSF samples of these patients.

To our surprise, we found that 7 % (18/258) of the cases diagnosed with TBM were infected with *M.bovis*, the classical causative agent of bovine TB, also reportedly responsible for human TB, which makes this bacterium an important zoonotic pathogen (Allix-Béguec, et al., 2010). In developed countries, the introduction of pasteurization and eradication programs for infected herds have considerably reduced the prevalence of human disease due to the bovine TB bacillus, but have not completely eradicated it (Evans, et al., 2007). In developing countries however, *M.bovis* has been reported to account for nearly 10 to 15% of new human TB cases (Ashford, Whitney, & Raghunathan, 2001).

The classical biochemical tests for the identification of *M.bovis* and molecular methods based on targets like IS6110, 16S rDNA, 23S rDNA or ITS cannot distinguish between *M.bovis* and the other members of the *M.tb*complex (MTBC). Other genetic markers and the single commercial test (GenoType Mycobacterium, Hain, Nehren, Germany) allowing distinction between MTBC members are not widely used due to the associated high costs (Sansila, et al., 1998). Diagnostically, the low sensitivity of CSF TB PCR is also problematic. Potential explanations for the lack of sensitivity in CSF specimens include low bacillary load in CSF, small sample volumes, and PCR inhibitors in the samples (Christie, et al., 2008). Moreover, from the clinician point of view, TB caused by *M.tb*. in humans is clinically and radiologically identical to TB caused by *M.bovis* (Grange, 2001).The distinction of *M.bovis* from *M.tb* however, has significant relevance to patient management since *M.bovis* is intrinsically resistant to pyrazinamide and the absence of specific identification may have adverse consequences for infected cases (Niemann, Richter , & Rüsch-Gerdes, 2000). Identification of this pathogen would thus help clinicians to adopt good patient management with respect to treatment regimen. Reports by Allix-Béguec, et al. (2010) and Hannan, et al. (2001) .have thus underscored the importance of routine use of molecular tests for differentiation of *M.bovis* from *M.tb* and/or systematic checking of resistance to pyrazinamide.

Earlier different studies by investigators showed the presence of *M.bovis* infection in humans (Kidane, et al, 2002; Wei, Huang JJ, & Chu , 1999; LoBue, Betacourt, & Peter C, 2003; Cosivi, et al., 1998). In the Indian context, a study by Jain (2011) demonstrated *M.bovis* infection in 9.52% cases and co-infection of both *M.tb* and *M.bovis* in4.76% cases through a two-step PCR targeting hup B gene . A study by Prasad, et al. (2005*)* reported 34.7% PCR positivity, of which *M.tb* was detected in 15.7%, *M.bovis* was seen in 10.3% and mixed infection in 8.7% of samples another study by Shah, et al. (2006) described the utility of a nested PCR (N-PCR) assay in detecting *M.tb*

and *M.bovis* in human CSF. They reported the presence of *M.bovis* in 17% cases and mixed infection in 22 %.

In the present study, of the total 258 cases, we detected *M.bovis* in 18 (7%) cases; of which two suffered fatal outcome. One case; a 24 year old pregnant woman (ANC 26 weeks), was admitted with typical symptoms of TBM including altered sensorium, intermittent fever, decreased appetite, vomiting and severe headache. Her CT scan showed meningeal enhancement, chest X-ray showed Koch's infiltrations and was subsequently started on anti-TB treatment. The patient delivered a healthy premature baby during the hospital course and was discharged. However, after 6 months, she again developed symptoms including abnormal behavior, marked neck-stiffness and drowsiness. The patient was readmitted and surgically treated. The patient had however expired before the 12 month follow-up. Regardless and more importantly, the initial absence of *M.bovis* identification of this patient's isolate compromised the efficiency of her treatments, and plausibly influenced the final fatal outcome.

Second case, a 68 year old male, had a history of right sided pyothorax (tubercular) and infarction in corona radiata was discharged against medical advice from a local hospital and consequently admitted to CIIMS. The patient upon admission was started with anti-TB treatment and was discharged on improvement. The patient showed neurological sequelae upon follow-up and was readmitted, however suffered septic shock and died of multi-organ failure. In the remaining *M.bovis* infected cases also, the standard treatment regimen proved to be ineffective leading to neurological deterioration with symptoms such as slurred speech, decreased hearing and difficulty in walking. Assessment of demographic characteristics of these individuals indicated that all the patients belonged to rural settings where agriculture was the main source of livelihood. All the subjects had occupational exposure to farm animals. Close physical contact with animals and

consumption of unpasteurized milk could be the potential sources of *M.bovis* transmission in these individuals. A report by Michel, Muller, & Van Helden (2010) has suggested that pastoralist and rural communities are at greatest risk for zoonotic TB, but the lack of data for these population groups prevents confirmation of this assumption.

While a number of articles have described the epidemiology, clinical features, and transmission of *M.bovis* disease in humans, little has been written about its treatment (LoBue & Moser, 2005). Beyond its use for specific *M.bovis* identification, this natural resistance is particularly important to consider. Pyrazinamide is usually given in the classical first-line TB treatment, as it is an effective sterilizing drug that helps to shorten TB therapy due to its synergistic effect with rifampicin. Thus, in case of *M.bovis* infection, pyrazinamide would be ineffective if implemented in a patient's anti-TB regimen. The duration of treatment thus tended to be longer for patients with *M.bovis* infection, as they were considered ineligible for a 6-month treatment regimen due to PZA resistance (LoBue & Moser, 2005). A study by LoBue *et al* also showed that the death rate was highest for *M.bovis* patients (LoBue & Moser, 2005). In the present study, given the high rates of *M.bovis* in TBM patients, surveillance of this zoonotic pathogen is obviated.

5. Conclusion

Our study demonstrates the prevalence of *M.bovis* induced TB in patients with poor outcomes. In addition, our report also constitutes an example of the persistent significance of *M.bovis* as a zoonotic pathogen. Human TB due to *M.bovis* is still underestimated, because of frequent use of diagnostic techniques that do not specifically distinguish *M.bovis* from other members of the *M. tuberculosis* complex, and because susceptibility to pyrazinamide is not systematically tested.

Finally, it demonstrates that molecular-guided cooperation between human and veterinary health services can improve detection of zoonoses in future.

Funding

This work was supported by the Indian Council of Medical Research (ICMR), New Delhi, India (Grant No. Zon.15/11/2014-ECD-II).

Conflicts of Interest

None

Acknowledgement

The authors acknowledge Dr. Dewanand R. Kalorey, Dr. Nitin V. Kurkure and Dr. Sandeep P. Chaudhari for their support. The authors would also like to thank Ms. Sonali Manke and Ms. Ankita Tiwari for their technical assistance.

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Legends

Table legends

Table 1: Primer sequences for RD region analysis.

Table 2. Classification of study participants

Table 3. Clinical characteristics of recruited participants

Table 4. Clinical and radiological findings of follow-up TBM cases

Table 5. Comparative efficiency of duplex PCR and Bactec culture for detection of mycobacteria.

Table 6. Drug susceptibility testing of positive culture isolate.

Figure legends

Figure 1: Schematic representation of the study design.

Figure 2: Molecular analysis for identification and differentiation of Mycobacterium bovis (M. bovis), Mycobacterium bovisBCG (M. bovisBCG) and Mycobacterium tuberculosis (M.tb) using duplex PCR. Position of the primers in the genomes is depicted.

Figure 3: Duplex PCR for detecting and differentiating M.bovis, M.tuberculosis, and M.bovis BCG. a) The ethidium bromide-stained amplification products of L1: M. bovis, L2: M. bovisBCG and L3: M. tuberculosis when electrophoresced on 2% agarose gel. The 176 bp and 110 bp products obtained are indicated. b) L1: 100 bp molecular ladder, L2: Positive control, L3 and L4: CSF samples with M.bovis infection.

Figure 4A: Alignment of M.bovis RD4 region and PCR product of duplex PCR targeting the RD4 region in clinical sample from TBM patient (C1). Figure 4B: Alignment of M.bovis RD1 region and PCR product of duplex PCR targeting the RD1 region in the same sample (C1). Sequence alignment done using CLC Sequence Viewer, Version 6.6.1.

Table 1

PCR	Primers	Sequence	Annealing Temp	Amplicon size	Reference
RD4	F	5'-AATGGTTTGGTCATGACGCCTTC-3'	58 °C	176 bp	Taylor GM et al., 2011
	R	5'-CCCGTAGCGTTACTGAGAAATTGC-3'			
RD1	F	5'-CCCTTTCTCGTGTTTATAGTTTGA-3'	60 °C	110 bp	Halse et al., 2011
	R	5'-GCCATATCGTCCGGAGCTT-3'			

Table 1: Primer sequences for RD region analysis.

Table 2. Classification of study participants

Category*	No. of cases
Definite TBM	24
Probable TBM	123
Possible TBM	77
Non -TBM	34

*Classification was done on the basis of clinical, radiological and CSF findings.

Table 3. Clinical characteristics of recruited participants

Characteristics	TBM cases (n = 224)	Non TBM cases (n = 34)	P value
Gender			
Male	134 (59.82)	30 (88.24)	**P = 0.0045**
Female	90 (40.18)	4 (11.76)	**P = 0.0045**
Median Age (In years)	37.19 ± 17.67	37.01 ± 16.51	P = 0.9584
Clinical data			
Headache	1655 (73.5)	13 (38.2)	**P = 0.0004**
Fever	134 (59.8)	10 (29.4)	**P = 0.0040**
Seizure	46 (20.5)	3 (8.8)	P = 0.1925
Altered consciousness	26 (11.6)	3 (8.8)	P = 0.8671
Altered sensorium	99 (44.2)	6 (17.6)	**P = 0.0104**
Hallucinations	6 (2.7)	2 (5.9)	P = 0.7796
Neck stiffness	123 (54.9)	4 (11.8)	**P < 0.0001**
Duration of hospital stay	30	15	
Radiological findings			
Hydrocephalus	22 (9.8)	2 (5.9)	P = 0.7299
Meningeal enhancement	70 (31.3)	6 (17.6)	P = 0.1828
Laboratory results			
Proteins	128.97	91.84	**P = 0.0322**
Glucose	31.93	60.38	**P < 0.0001**
Cells	209.72	83.75	**P = 0.0003**

Table 4. Clinical and radiological findings of follow-up TBM cases.

Characteristics	Follow-up * 6 months	Follow-up # 12 months
Clinical findings		
Neurological Sequelae/Deterioration	65 (36.1)	16 (15.7)
Death	18 (10)	2 (2)
Improvement	97 (53.9)	84 (82.3)
Radiological findings		
Hydrocephalus	41 (22.8)	5 (4.9)
Meningeal enhancement	7 (3.9)	13 (12.7)
Stroke	1 (0.6)	0 (0)
Laboratory findings		
Proteins>100 mg%	48¶(26.7)	18¶ (17.6)
Glucose <2/3 CBS	23¶ (12.8)	15¶ (14.7)
Cells>20 cells/mm^3	34¶ (18.9)	13¶ (12.7)

* n = 180 (44 cases lost to follow-up)
#n = 102 (18 cases expired, lost to follow-up)
¶ CSF was collected from patients with neurological deterioration.

Table 5. Comparative efficiency of duplex PCR and Bactec culture for detection of mycobacteria.

Duplex PCR result	**PCR result No. (%)**	**Species identified**		**Culture result No. (%)**	
		***M.tuberculosis* No.(%)**	***M.bovis* No. (%)**	**Positive**	**Negative**
Positive	103 (39.9)	85 (32.9)	18 (7.0)	28(27.2)	75 (72.8)
Negative	155 (60.1)	173 (67.1)	240 (93.0)	8 (5.2)	147 (94.8)
Total	258 (100)	258 (100)	258 (100)	36 (14)	222 (86)

Table 6. Drug susceptibility testing of positive culture isolate.

Antitubercular Drug	Critical Concentration (µg/ml)	Interpretation
Streptomycin	1	Susceptible
Isoniazid	0.1	Susceptible
Rifampicin	1	Susceptible
Ethambutol	5	Susceptible
Pyrazinamide	100	Resistant

Figure 1

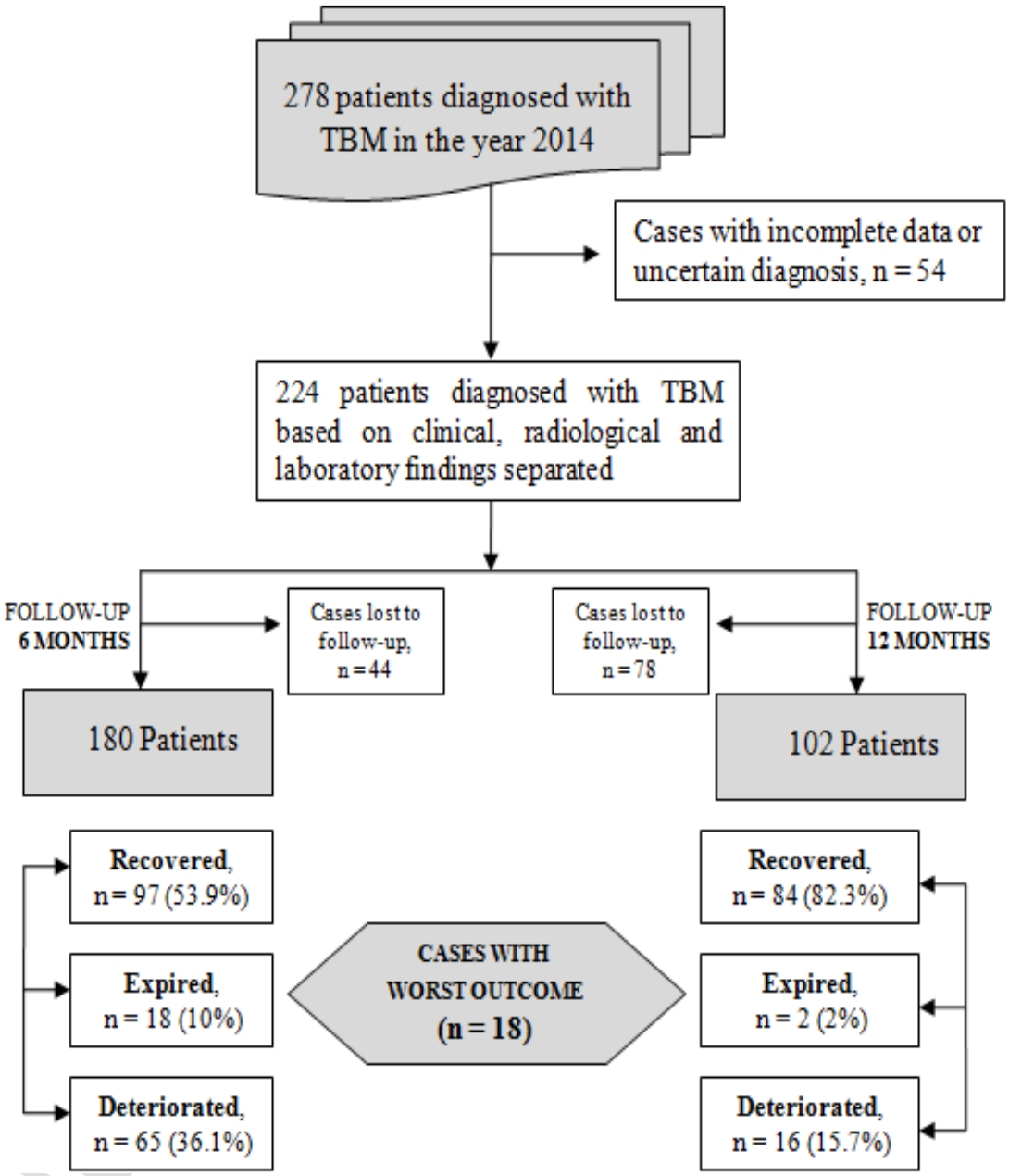
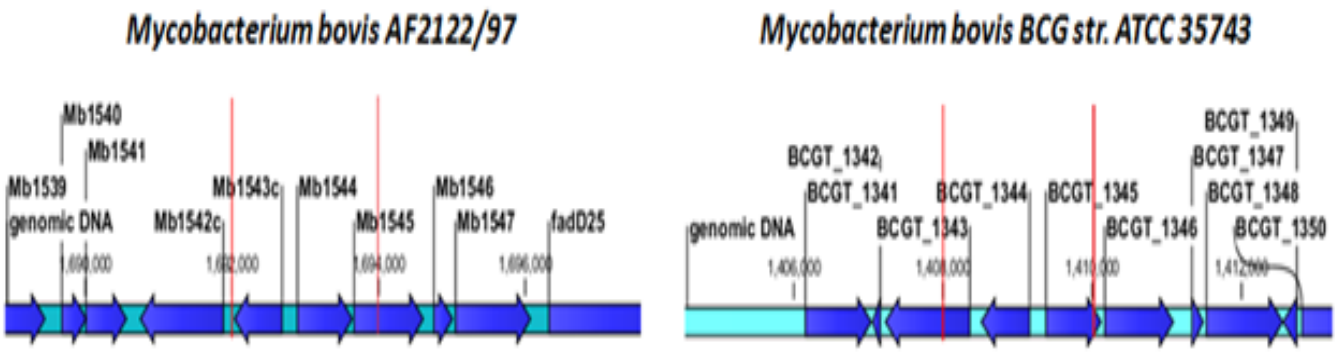
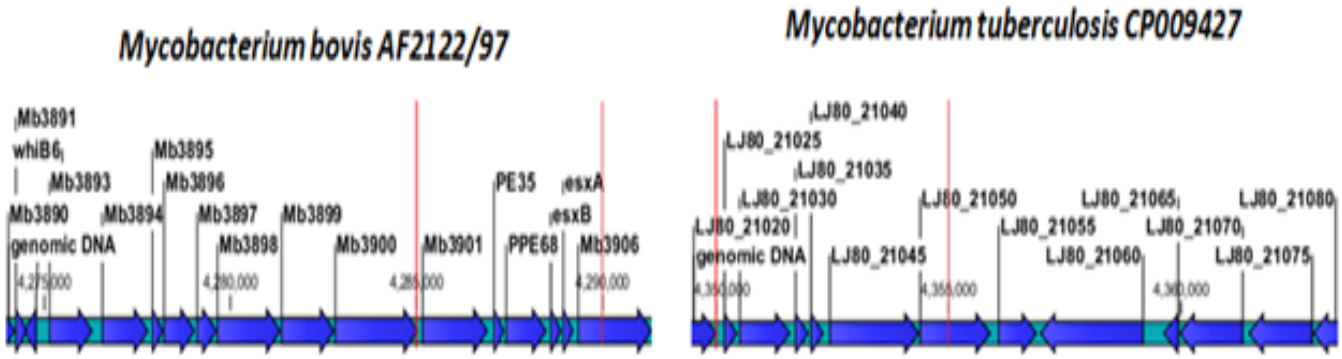


Figure 2



RD4 region of *M.bovis* and *M.bovis* BCG genomes giving 176-bp amplification product.



RD1 region of *M.bovis* and *M.tuberculosis* genomes giving 110-bp 176-bp amplification product.

Figure 3

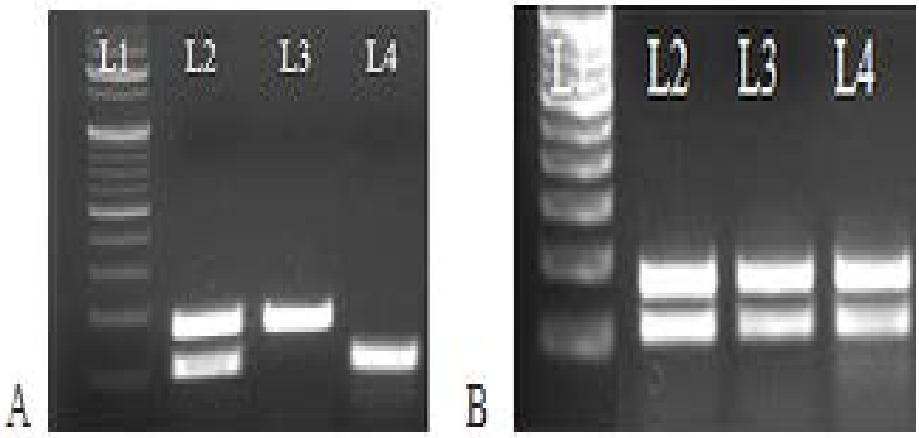
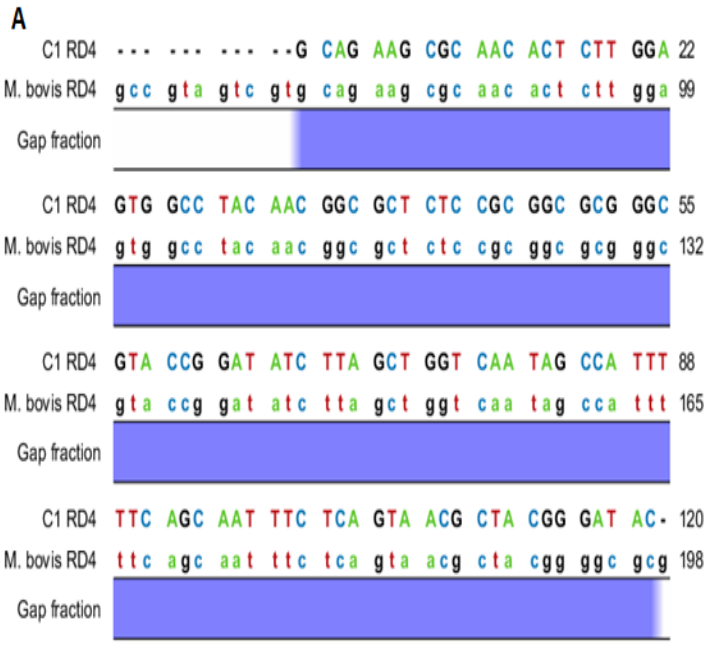
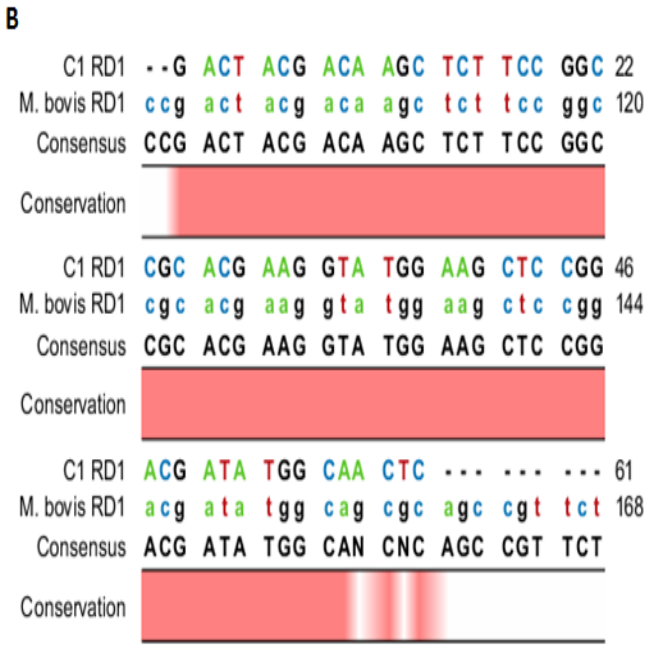


Figure 4


A
C1 RD4 --- --- --- --G CAG AAG CGC AAC ACT CTT GGA 22
M. bovis RD4 gcc gta gtc gtg cag aag cgc aac act ctt gga 99
Gap fraction
C1 RD4 GTG GCC TAC AAC GGC GCT CTC CGC GGC GCG GGC 55
M. bovis RD4 gtg gcc tac aac ggc gct ctc cgc ggc gcg ggc 132
Gap fraction
C1 RD4 GTA CCG GAT ATC TTA GCT GGT CAA TAG CCA TTT 88
M. bovis RD4 gta ccg gat atc tta gct ggt caa tag cca ttt 165
Gap fraction
C1 RD4 TTC AGC AAT TTC TCA GTA ACG CTA CGG GAT AC- 120
M. bovis RD4 ttc agc aat ttc tca gta acg cta cgg ggc gcg 198
Gap fraction


B
C1 RD1 --G ACT ACG ACA AGC TCT TCC GGC 22
M. bovis RD1 ccg act acg aca agc tct tcc ggc 120
Consensus CCG ACT ACG ACA AGC TCT TCC GGC
Conservation
C1 RD1 CGC ACG AAG GTA TGG AAG CTC CGG 46
M. bovis RD1 cgc acg aag gta tgg aag ctc cgg 144
Consensus CGC ACG AAG GTA TGG AAG CTC CGG
Conservation
C1 RD1 ACG ATA TGG CAA CTC --- --- --- 61
M. bovis RD1 acg ata tgg cag cgc agc cgt tct 168
Consensus ACG ATA TGG CAN CNC AGC CGT TCT
Conservation