Appendix A: 16S rRNA sequence

Appendix A.1: 16S rRNA sequence of *M. avium*

3'TGCGAAACACAGCCGCCTATATAGCAGTGAAGACGGCCGAGTTAGCTGCTTTTCCCTGCGGGCATAATTGTGCTGGGGGGAAGA
CGCCTGATACCCCAGGTTTCTCCGGGAGGAGGAGGATATATCAAACAATCCATAAAACCGTATATGATTATGGATCTTGATTTAAAGGGTGGAAATATCTCAATGCAACGTTGATGTATGGACCTGCATTGTATTTGTGGGGACGTGGGGCAACGGCGCGGCTACGCAAACGCCGCTTGCCGAGGTGATCGGCCACTCTGCCACTCTGACACCGCAAACACGCCCAGACGGCGACGGTAGGGGAAATTTTCGAATGTACGGGAATCCGGGAAAGCCCAACCCCGCGAGTGTAAAGAAGTTTAGAATATTTTAGCTTTGTTTTGTTATAGTATAAAGAGTGAGAGAGTGGAATTTTCACCGTGTCATACTGTGACACCACAGAACCAGGACAGAACGCCTCACCACCCGCCCTCAGACACGTATGTCCATAACTCTCGAGAGATATCTTGAGCGAAAAGAGCGCACGCGGGGTCAGTAAATTCTGATATTAAGAACTGTATCGCACCTCCTGCACTCTTGAAAGTCTTTTAAACTAGTGCACTAAAGGAGAGAGTGGAATAGTGTATTTTCCTGTGATGCGTTTATATGTATAGATGAACACCGGGAAGAGAACGCGGCTATGTGGGTGTTATTGTCTGTAACTGACTCGAACGCTTGGAGAGCAGAGGGAGAGGATACGCGGGGGGTGCCCCGCGCGACCAAACCAGAGCGCTAGGGTTGGGGGGCTCTTTGGGTCGTTTTCCGTGCCGCCTTTCACCTAACTAACTTATCCCCCCGGGGGGACCGGGCCCGGAGGGTAGAAAACGTTTAAAGATTAAAATGGATGCCCCCCAAGCCCTGGCGGACCATGTGTTTATTATTTCTTTAAAATCCCAAGAAAACCCATACCAGGTTTACGACGTCCCGGCATCCCTT 5'

Appendix A.2: 16S rRNA sequence of *M. bovis*

3'GAGAAGAGCTGGCGGGCGTGCCTATCATACATCGATCTAAGGAAAGAAGCTCTTCTTCTCCTACTGGAATAGAAGAAGGCGGAGGAGTGATGAGTAACGCGTAATCATCCTGCCCTTCCCAGGGGGACCCCTTATTGGTAACGACTGCTATTACCGCATACGATTTACCTCGGGTTTGATGATTGATGAAAGGAGGCCTCTACTGTAAGCTATCACTGAGGGACGGCATTGTGTCTGATAAGTTAGTTGGACGGGTACCAGCCCACCAAGGCGAAGATCACTAGCTGGTCTGACAATCTGAAACTTTGCATTGGAACTGAGACCCAGCCTACACTCCTACGGGGGGCCTTGGGAAGTGAGGATATGCATGAGAGACAATCTGACGTGGCCGCCCCCACTT5'

Appendix A.3: 16S rRNA sequence of *M. tuberculosis*

3'AGGTATCCGACGCTGAAAAAAGGGGTTGCTTTCTTGGGACTATTTGAGACGAGGCGGTGCTGTAACGAGCTTTCTGCAACCCCCCGGCCTTTCTCCGGGAGGCCCTTGTGAACTCTAGCAAACAATCCATAAAACCGTATATGATTATGGATCTTGATTTAAAGGGTGGAAATATCTCAATGCAACGTTGATGTATGGACCTGCATTGTATTTGTGGGGACGTGGGGCAACGGCGCGGCTACGCAAACGCCGCTTGCCGAGGTGATCGGCCACTCTGCCACTCTGACACCGCAAACACGCCCAGACGGCGACGGTAGGGGAAATTTTCGAATGTACGGGAATCCGGGAAAGCCCAACCCCGCGAGTGTAAAGAAGTTTAGAATATTTTAGCTTTGTTTTGTTATAGTATAAAGAGTGAGAGAGTGGAATTTTCACCGTGTCATACTGTGACACCACAGAACCAGGACAGAACGCCTCACCACCCGCCCTCAGACACGTATGTCCATAACTCTCGAGAGATATCTTGAGCGAAAAGAGCGCACGCGGGGTCAGTAAATTCTGATATTAAGAACTGTATCGCACCTCCTGCACTCTTGAAAGTCTTTTAAACTAGTGCACTAAAGGAGAGAGTGGAATAGTGTATTTTCCTGTGATGCGTTTATATGTATAGATGAACACCGGGAAGAGAACGCGGCTATGTGGGTGTTATTGTCTGTAACTGACTCGAACGCTTGGAGAGCAGAGGGAGAGGATACGCGGGGGGTGCCCCGCGCGACCAAACCAGAGCGCTAGGGTTGGGGGGCTCTTTGGGTCGTTTTCCGTGCCGCCTTTCACCTAACTAACTTATCCCCCCGGGGGGACCGGGCCCGGAGGGTAGAAAACGTTTAAAGATTAAAATGGATGCCCCCCAAGCCCTGGCGGACCATGTGTTTATTATTTCTTTAAAATCCCAAGAAAACCCATACCAGGTTTACGACGTCCCGGCATCCCTT 5'

Appendix A.4: 16S rRNA sequence of *M. microti*

3'GGGTATCCAAAACGCATGAAAAGATGGGGTTGCTTTCTTGGGACTATTTGAGACGAGGCGGTGCTGTAACGAGCTTTCTGCAACCCCCCGGCCTTTCTCCGGGAGGCCCTTGTGAACTCTAGCAAACAATCCATAAAACCGTATATGATTATGGATCTTGATTTAAAGGGTGGAAATATCTCAATGCAACGTTGATGTATGGACCTGCATTGTATTTGTGGGGACGTGGGGCAACGGCGCGGCTACGCAAACGCCGCTTGCCGAGGTGATCGGCCACTCTGCCACTCTGACACCGCAAACACGCCCAGACGGCGACGGTAGGGGAAATTTTCGAATGTACGGGAATCCGGGAAAGCCCAACCCCGCGAGTGTAAAGAAGTTTAGAATATTTTAGCTTTGTTTTGTTATAGTATAAAGAGTGAGAGAGTGGAATTTTCACCGTGTCATACTGTGACACCACAGAACCAGGACAGAACGCCTCACCACCCGCCCTCAGACACGTATGTCCATAACTCTCGAGAGATATCTTGAGCGAAAAGAGCGCACGCGGGGTCAGTAAATTCTGATATTAAGAACTGTATCGCACCTCCTGCACTCTTGAAAGTCTTTTAAACTAGTGCACTAAAGGAGAGAGTGGAATAGTGTATTTTCCTGTGATGCGTTTATATGTATAGATGAACACCGGGAAGAGAACGCGGCTATGTGGGTGTTATTGTCTGTAACTGACTCGAACGCTTGGAGAGCAGAGGGAGAGGATACGCGGGGGGTGCCCCGCGCGACCAAACCAGAGCGCTAGGGTTGGGGGGCTCTTTGGGTCGTTTTCCGTGCCGCCTTTCACCTAACTAACTTATCCCCCCGGGGGGACCGGGCCCGGAGGGTAGAAAACGTTTAAAGATTAAAATGGATGCCCCCCAAGCCCTGGCGGACCATGTGTTTATTATTTCTTTAAAATCCCAAGAAAACCCATACCAGGTTTACGACGTCCCGGCATCCCTT 5'
Appendix A.4: 16S rRNA sequence of *M. scrofulaceum*

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TCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGGGCAGCAGTAGGGAATCTTCGGCAATGGAGGGAACTCT
GACCGAGCAACCCGAGGGGGTCTAATAATGAGTGGGCTTCTCTTTTGGGGATTCAGTGCATACn'ACACAAAAAGCACCCCCCC
GGGGGGAGTACGACCGCGGTGTAGAATCTAAAGGGGATTCGACGGGGGGCCCCGCACAAAGCGGGGAGAGCATGTGTTTTTTAT
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CAACGCGATGGAGGTGGCGGCGGCTATATATAGCCTGCCAGGAGGAGGAGAGGAGGGAGGGGACCTGGCGGGGTAGCGGGAG
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TCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGGGCAGCAGTAGGGAATCTTCGGCAATGGAGGGAACTCT
GACCGAGCAACCCGAGGGGGTCTAATAATGAGTGGGCTTCTCTTTTGGGGATTCAGTGCATACn'ACACAAAAAGCACCCCCCC
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Appendix A.5: 16S rRNA sequence of *M. africanum*

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TTAAGAAGACACTGCGAGACACTGCGAGACACTGCGAGACACTGCGAGACACTGCGAGACACTGCGAGACACTGCGAGACACT
Appendix A.6: 16S rRNA sequence of *M. terrae*

3'GCAGTAACCTGGGGGGCTCTCATATATGAGTGAGTGGGCTTCTCTTTTGGGGATTCAGTGCATACn'ACACAAAAAGCACCCCCCC
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Appendix A.7: 16S rRNA sequence of *M. flavescens*

3'CGGCAAGGGGGGCTCCTAC/CATCGAGTAGAAGCTGAAGGGTTGAGGTGCTTGTGCTCTTCGATGTAAGTTGGAACGCGTGAT
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ACCATTACCGGGGTCGTACCTCCGCTTCTACAGCAGCTCTA 5'
Appendix B: Publications

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Analysis of mutational pattern in multidrug resistant tuberculosis (MDR TB) in a geographically isolated northeastern region of India

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Abstract: Objectives: To assess the mutational pattern of multidrug resistant tuberculosis and evaluate the performance of MTBDRplus and MTBDRsl in rapid detection of multidrug and second line resistance of tuberculosis in a geographically isolated population.

Methods: The assay was performed directly on 375 smear positive sputum specimens from patients referred to a busy routine diagnostic lab in Manipur, northeast India. MTBDRplus and MTBDRsl were performed as per the directions of the manufacturer. Results were compared with BacT/Alert 3D culture and DST system.

Results: Of the 375 smear positive specimen, 364 (97.1%) gave interpretable results by MTBDRplus assay within 1 – 2 days with an additional 2 days for EMB resistance of the MDR strains. Sensitivity, specificity, and positive and negative predictive values were high for rifampicin and multidrug resistant strains with a range of 95 – 100 %. Isoniazid has comparatively lower sensitivity of 75% amongst the drug compared with phenotypic BacT/Alert 3D results. The mutational pattern of this geographically isolated region didn't have much variation as shown by the absence of bands in the rpoB M_172A/2B and inhA M_3A/3B regions of the gene.

Conclusions: The results of this study supports the use of MTBDRplus for rapid diagnosis of TB from smear positive specimen considering the drastic reduction of time in diagnosis and its accuracy being comparable to phenotypic methods while the MTBDRsl's ability to detect rare mutations makes it a potential method for analyzing mutational patterns.

Keywords: tuberculosis; MTBDRplus; MTBDRsl; northeast India; Manipur

1. Introduction:
India has become the world hub for tuberculosis. According to the latest World Health Organisation (WHO) report titled “Global Tuberculosis Control 2011” >1.8 million cases of TB are reported of which 2.5 million were from India. India alone accounted for an estimated 26% of all TB cases worldwide and remains a major killer, killing 2 persons every 3 minutes which is nearly 1000/day.

Multidrug resistant tuberculosis (MDR TB) has evolved to become a serious threat to the general public due to limited treatment options (Ching et al, 2006; Morgan et al, 2005). In order to have efficient treatment, it becomes imperative that detection be done as early as possible. The emergence of extensively drug resistant tuberculosis (XDR TB) looms large in the backdrop of such scenario with further spread of drug resistance, especially in HIV-infected patients, as it was recently reported (Grandi2006). XDR TB, defined as MDR TB with additional resistance to fluoroquinolone antibiotic and at least one of the three injectable drug used for MDR TB treatment (Cenere 1 & 2) has been identified in 84 countries with an average proportion of MDR TB cases with XDR TB as 9% (6.7 – 11.2%) (WHO, 2012).

As such early detection of drug resistances constitutes one of the priorities of TB control programs. Detection of drug resistance has been performed in the past by conventional methods based on detection of growth of M. tuberculosis in the presence of antibiotics. Conventional indirect methods of detection for drug resistance take 10 – 12 weeks for the sample to get drug susceptibility test (DST) report while direct method may be given by 4 – 6 week (Canetti et al, 1963; Caviedes et al, 2002; Kent & Kubica, 1985). However due to the labouriousness of some of this methods and most of all the long period of time necessary to obtain results, new technologies and approaches have been proposed which include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of rifampicin (RIF) resistance, since it is considered a good surrogate marker for MDR TB, especially in settings with high prevalence of MDR TB (Ching et al, 2006; Morgan et al, 2005). Genotypic methods have the advantage of a shorter turnaround time, no need for culture of organism, possibility of direct application in clinical samples, lower biohazard risk and the feasibility of automation. However, not all mechanism of drug resistance are known. Genotypic methods for drug resistance look for the genetic determinants of resistance rather than the
Analysis of mutational patterns in multidrug resistant tuberculosis (MDR TB) in a geographically
resistant phenotype and involve two basic steps - nucleic acid amplification by polymerase chain reaction
(PCR) to amplify the sections of the M. tuberculosis genome known to be altered in resistant strains and a second
step of assessing the amplified products for specific mutations correlating with drug resistance (Garcia de
Viedma, 2003; Palomino, 2005). Nearly all RIF resistant strains contain mutation of the rpoB gene while
mutations in the emhB gene were associated with ethambutol (EMB) resistance (Cole, 1996; Riccardi et al,
2009). Mutations in katG gene and inha gene were related to high level and low level isoniazid (INH)
resistance respectively (Vliegen et Jacob, 2007).

The nature and frequency of mutations in the resistant strains vary significantly based on the
geographical location (Mokrousov et al, 2002). There is very less information available on specific mutational
patterns in India (Sharma et al, 2003), let alone on the underdeveloped and isolated region of northeast India.
MDR TB strains have been reported mostly from countries where HIV and TB co-infection is endemic which
includes India (Prasad, 2005). The dual challenges in TB and HIV co-infections are particularly pressing
in Manipur, a state in the easternmost corner of northeast India which has the highest estimated adult HIV
prevalence in India (NACO India, 2012).

The present study is being undertaken with an aim to extend the knowledge of mutational pattern of
M. tuberculosis complex by using the MTBDRplus and MTBDRs/d (Hain LifeScience) in Manipur, a
geographically isolated region of northeast India. The study also evaluates the MTBDR method by comparing
with phenotypic culture based method (BacT Alert 3D) on an attempt to highlight the feasibility of applications
in the current situation of TB, its drug resistance and diagnostic trends in northern India.

II. Material And Methods

Study settings.

This study was conducted at the Babina Diagnostic Centre in Imphal, Manipur which is one of the most
prominent and main referral diagnostic lab of the region. It currently serves a population of 2.7 million.
Testing was performed on residual portions of routine clinical specimens submitted for culture and DST.
Informed consent was not taken for the study as results were unlinked from patient identifiers and no patient
information was collected. Only one sample per patient was collected.

Sputum specimens

Three hundred and seventy five sputum samples were collected between May – December 2011. Every
handling of the clinical specimens was conducted inside a Class II safety cabinets in BSL2 laboratory in
accordance with CDC guidelines. Sputum decontaminations were carried out with the conventional
N-acetyl-L-cysteine-NaOH. After centrifugation, the pellet was suspended in 1ml of 5% NaCl. All specimens were
processed for acid fast microscopy using Ziel-Neesen technique (Caietti et al. 1963; Kent & Kubica, 1985).
Sputum samples showing more than 10 acid fast bacilli (AFB) per microscopic field in the smear were selected
for the study.

BacT/Alert 3D culture and DST

Drug susceptibility testing for isoniazid, rifampicin and ethambutol was performed by BacT/Alert 3D
system. A 0.5ml portion of the sediment was inoculated into the vials of BacT/Alert 3D system containing
modified Middlebrook 7H9 media with respective antibiotic. The final drug concentration in the test bottles was
typical for INH and RIF and 25ug/ml for EMB. All mycobacterial cultures were incubated at 37°C and were
continuously monitored. M. tuberculosis isolate was determined to be resistant to the antibiotic when the drug
containing bottle had a time to detection (TTD) that was less than or equal to the TTD of 1% control.

Genotypic MTBDRplus and MTBDRs/d DST

The MTBDRplus and MTBDRs/d DST were performed according to the manufacturer’s instruction and
divided into three steps: DNA extraction, a multiplex amplification using biotinylated primers and reverse
hybridisation. A 500μl portion of the decontaminated sediment was used for DNA extraction using modified
CTAB-NaCl method. The isolated DNA samples from the process mentioned above were used for multiplex
amplification with biotinylated primers. The biotin labelled PCR product was chemically denatured and
hybridised in the strip with specific oligonucleotide probes. After hybridisation and washing, strips were
removed, allowed in dry and fixed on the nitrocellulose paper.

All samples that tested positive for MDR in the MTBDRplus strip was further subjected to MTBDRs/d test.
Phenotypic DST was conducted for EMB only in the MDR strains which has been subjected MTBDRs/d. All
tests were performed independent of culture and DST.

Interpretation of results

Each strip in MTBDRplus consist of 27 reaction zones (bands) including six controls (comparate, assimilation, M. tuberculosis complex, rpoB, katG and inha controls), eight rpoB wild type (WT) and four
mutant (MUT) probes, one katG wild type and two mutant and two inha wild type and four mutant probes
Analysis of mutational pattern in multidrug resistant tuberculosis (MDR TB) in a geographically

(Figure 1a). Each strip in the MTBDRplus consists of 22 reaction zones including six controls (conjugate, amplification, M. tuberculosis complex, gyrA, rrs and emaB controls). Three gyrA wild type and six mutant probes, two rrs wild type and two mutant probes and one emaB wild type and two mutant probes (Figure 1b).

Results were interpreted according to the manufacturer's instructions.

III. Results

Genotype MTBDRplus testing from smear positive specimens

Genotype MTBDRplus test results in comparison with BacT/Alert 3D DST of all smear positive specimens tested (n = 375) are summarised in Table 1. Fifteen specimens (4%) were culture negative and therefore no phenotypic DST results were available. Of these 15 culture negative specimens, 14 (93.33%) gave interpretable results by the MTBDRplus method. Of the specimens with conventional DST results, 60 (16.67%) were NDR, 20 (5.6%) were RIF monoresistant, 14 (4.44%) were INH monoresistant and 366 (73.33%) were RIF resistant with one uninterpretable result out of the 60 MDR strains detected by phenotypic DST. The results for RIF monoresistant was concordant with the phenotypic DST except for one strain which was detected as RIF and INH susceptible out of the 20 RIF monoresistant by phenotypic DST. MTBDRplus detected 369 susceptible strains including 13 culture negative strains while BacT/Alert system could detect 264 susceptible strains.

Table 3 shows the distribution of different banding patterns in drug resistant isolates, including MDR, INH monoresistant and RIF monoresistant strains while Table 4 shows the banding pattern of MDR isolates which has been subjected to MTBDRplus test. Typical banding patterns obtained on MTBDRplus and MTBDR strips are shown in Figure 1.

The RIF resistant isolates displayed different mutations. The most common mutation was in the S331L region (MUT3) with 45.6% of all RIF resistant strains (55% of MDR and 19% of RIF monoresistant strains) having the mutation in the katO gene probe while mutation was detected in all the mutational probes of gyrA gene with highest rate in the D94G region (17%). One MDR positive strain had a gyrA MUB3 mutation. This is a rare mutation which has been detected only in silico and hence thought to be undetectable in vitro. But the detection in one strain proves that it is detectable and that the MTBDRplus is efficient in detecting even rare mutations. Of the 32 EMB resistant strains, 27 strain had mutations in the embA gene region of codon 306 with amino acid change of Met-306Val and 27 strain had mutation in the emaB gene with amino acid change of Met-306Val. One strain had mutation that was detected only in the wild type probes (emaB WT) but not at the mutant probes.

Performance of Genotype MTBDRplus and Genotype MTBDRplus assays

www.iosrjournals.org 6 | Page
Analysis of mutational patterns in multidrug resistant tuberculosis (MDR TB) in a geographically

Performance parameters for detection of INH, RIF monoresistance, MDR and EMR resistance were calculated from specimens for which genotypic and phenotypic DST results were available (Table 2). The performance of the genotypic MTBDRplus test directly from smear positive sputum correlated highly with the phenotypic Bact/Alert automated DST. Compared with the phenotypic DST, the genotypic MTBDRplus had a sensitivity of 81.3%, specificity of 99.2% and accuracy of 98.2% for detection of INH, a sensitivity of 95%, specificity of 100% and accuracy of 94.6% for RIF resistance and a sensitivity of 98.3%, specificity of 100% and accuracy of 99.7% for MDR. The genotypic MTBDRplus assay for ethambutol had a sensitivity of 86%, specificity of 91% and accuracy of 88% for detection of INH resistance.

IV. Discussion

The efficiency of Genotype MTBDRplus has been reported by many authors (Hilleman et al, 2006, 2007; Barnard et al, 2007, 2008) having good concordance with phenotypic DST results but presence of false negative due to unique mutation are also reported (Hilleman et al, 2007; Brossier et al, 2006; Tesema et al, 2012). This may be due to specific location independently developing endemic mutations. The present study evaluates the INH, RIF, MDR and EMR mutations using genotypic MTBDRplus and MTBDRplus test compared with Bact/Alert 3D DST of a geographically isolated region of northeast India. The results of the study showed that the mutational pattern of the resistant genes didn’t vary much with most mutations confined to one or two main regions. It is seen that in this region of India, the most frequent mutation causing RIF resistance is the Ser315Leu mutation (4.6%) and in INH resistance, the Ser315Thr is the most common (19%). This is more or less in agreement with earlier reported studies (Miotto et al, 2006; Mokrousov, 2002). In case of INH resistance, Mct306 Val mutation (45%) is observed to be the major cause. Previous studies have shown that 40-95% of isoniazid resistance are attributed to katG mutations. In this present study, 77% of INH resistance are attributed to katG mutations of which 95% are S315T1 and 5% are S315T2 mutations. A high prevalence of katG mutations has been reported to account for a high proportion of INH resistance in high TB prevalent countries presumably due to ongoing transmission of these strains in high burden settings (Mokrousov et al, 2002). India, being one of the top high TB burden countries, such high prevalence was expected.

The performance characteristics of specificity, sensitivity, accuracy, PPV and NPV as given in Table 2 suggest that the MTBDRplus assay is equivalent to phenotypic culture based DST performed in quality assured reference laboratories but detection of ethambutol resistance by MTBDRplus, though lower than INH or RIF detection by MTBDRplus, is comparatively higher than other reported studies (Brossier et al, 2010; Tesema et al, 2012). The lower value of sensitivity for detection of INH resistance (81.25%) by genotypic method might be due to resistance inferred by mutations in other genomic region which has not been incorporated in this assay, such as inhA and inhB gene (Lee et al, 1999, 2001; Rindi et al, 2005). Three out of 16 INH monoresistant strains were detected by mutations in inhA gene only. These mutations would not have been detected by the previous MTBDR which didn’t incorporate the inhA probe. As such, mutations in other genomic regions might be inducing resistance to INH which would not be detected by genotypic method if their corresponding probes were not included. Leading to false positives apart from the false negative results due to presence of inhibitors (Palomino, 2006).

Identification and drug resistance assay of M. tuberculosis normally requires several weeks as they are very slow in their growth and even with automated culture system, it takes an average of 14 days. Another 14-30 days for additional tests are required for DST. Moreover, the culture based methods involve increased consumption of exclusive culture media and hence place more economic burden on patients especially in a low resource and high burden region like Manipur. On the basis of FIND-negotiated prices, the cost of molecular assay at less than 50% of that for conventional liquid culture and DST for INH and RIF (Barnard et al, 2008) therefore there is urgency for an efficient method of TB testing as a complement to conventional culture and smear microscopy.

In conclusion, the results of this study supports the use of MTBDRplus for rapid diagnosis of TB considering the drastic reduction of time in diagnosis and its accuracy being comparable to conventional methods. It might also be more cost effective in the long run. This molecular genotyping will also be useful in studying epidemiological and mutational analysis of M. tuberculosis of specific regions.

References


www.iosijournals.org 7 | Page
Tabic 1. Results of MDR by Genotype MTBDRplus in comparison with Bact/Alert culture and DST.

<table>
<thead>
<tr>
<th>Genotype MTBDRplus</th>
<th>MDR</th>
<th>RIF monoresistant</th>
<th>INH monoresistant</th>
<th>RIF &amp; INH susceptible</th>
<th>Culture negative</th>
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Definition of abbreviation: DST - Drug Susceptibility Test; RIF - Rifampicin, INH - Isoniazid, MDR - Multidrug resistant.

### Analysis of mutational pattern in multidrug resistant tuberculosis (MDR TB) in a geographically

#### Table 2. Performance of MTBDRplus in detecting MDR from smear positive sputum specimens

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rif 95 (9.0 - 97.0)</th>
<th>INH 94.2 (79.0 - 98.3)</th>
<th>Dr 98.3 (97.3 - 99.5)</th>
<th>Any 85.7 (82.6 - 88.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100 (99.0 - 100)</td>
<td>99.3 (98.4 - 99.9)</td>
<td>100 (99.0 - 100)</td>
<td>91.3 (88.8 - 93.8)</td>
</tr>
<tr>
<td>Specificity</td>
<td>99.6 (98.1 - 100)</td>
<td>98.1 (97.0 - 99.3)</td>
<td>99.6 (99.1 - 100)</td>
<td>87.9 (85.0 - 90.3)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.6 (98.1 - 100)</td>
<td>98.1 (97.0 - 99.3)</td>
<td>99.6 (99.1 - 100)</td>
<td>87.9 (85.0 - 90.3)</td>
</tr>
<tr>
<td>PPV</td>
<td>100 (99.0 - 100)</td>
<td>99.7 (99.0 - 100)</td>
<td>99.7 (99.0 - 100)</td>
<td>92.5 (91.6 - 95.9)</td>
</tr>
<tr>
<td>NPV</td>
<td>99.6 (99.1 - 100)</td>
<td>99.1 (98.6 - 99.6)</td>
<td>99.6 (99.1 - 100)</td>
<td>90.1 (85.0 - 95.0)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: NPV = negative predictive value; PPV = positive predictive value. Values are percentages with 95% confidence interval in parentheses.

#### Table 3. Pattern of gene mutations in resistant *M. tuberculosis* strains using Genotype MTBDRplus assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rand</th>
<th>Gene region of mutation</th>
<th>MDR (n = 58)</th>
<th>TINH Monoresistant (n = 16)</th>
<th>Rif Monoresistant (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td>WT1</td>
<td>505-509</td>
<td>54 (93)</td>
<td>16 (100)</td>
<td>19 (66)</td>
</tr>
<tr>
<td></td>
<td>WT2</td>
<td>510-513</td>
<td>54 (93)</td>
<td>16 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td></td>
<td>WT3</td>
<td>511-517</td>
<td>49 (84)</td>
<td>16 (100)</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>WT4</td>
<td>516-519</td>
<td>54 (93)</td>
<td>16 (100)</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>WT5</td>
<td>516-522</td>
<td>50 (87)</td>
<td>16 (100)</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>WT6</td>
<td>520-525</td>
<td>49 (84)</td>
<td>16 (100)</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>WT7</td>
<td>526-529</td>
<td>49 (84)</td>
<td>16 (100)</td>
<td>19 (61)</td>
</tr>
<tr>
<td></td>
<td>WT8</td>
<td>530-533</td>
<td>13 (22)</td>
<td>16 (100)</td>
<td>16 (100)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>F516V</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2A</td>
<td>H526Y</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2B</td>
<td>H526Y</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2C</td>
<td>H526Y</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT3</td>
<td>S531L</td>
<td>32 (55)</td>
<td>0 (0)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>katG</td>
<td>WT</td>
<td>315</td>
<td>13 (22)</td>
<td>2 (13)</td>
<td>2 (13)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>S215T</td>
<td>41 (71)</td>
<td>13 (81)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2</td>
<td>S215T</td>
<td>2 (3)</td>
<td>1 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>inhA</td>
<td>WT1</td>
<td>-151-156</td>
<td>47 (81)</td>
<td>13 (81)</td>
<td>21 (100)</td>
</tr>
<tr>
<td></td>
<td>WT2</td>
<td>-</td>
<td>55 (95)</td>
<td>15 (94)</td>
<td>21 (100)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>C157T</td>
<td>11 (19)</td>
<td>3 (16)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2</td>
<td>A160</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT3A</td>
<td>TBC</td>
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<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT3B</td>
<td>LBA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Rif = rifampicin; TINH = isoniazid; MDR = multidrug resistant; Values are numbers, with percentages in parentheses.

#### Table 4. Pattern of gene mutations in MDR *M. tuberculosis* strains using Genotype MTBDRsl assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rand</th>
<th>Gene region of mutation</th>
<th>Pattern of mutation (n = 58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>WT1</td>
<td>85-90</td>
<td>58 (90)</td>
</tr>
<tr>
<td></td>
<td>WT2</td>
<td>84-85</td>
<td>51 (88)</td>
</tr>
<tr>
<td></td>
<td>WT3</td>
<td>82-84</td>
<td>44 (75)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>A82V</td>
<td>11 (14)</td>
</tr>
<tr>
<td></td>
<td>MUT2</td>
<td>S81P</td>
<td>3 (5)</td>
</tr>
<tr>
<td></td>
<td>MUT3A</td>
<td>794A</td>
<td>5 (9)</td>
</tr>
<tr>
<td></td>
<td>MUT3B</td>
<td>794N/V</td>
<td>5 (9)</td>
</tr>
<tr>
<td></td>
<td>MUT3C</td>
<td>944G</td>
<td>10 (17)</td>
</tr>
<tr>
<td></td>
<td>MUT3D</td>
<td>9446</td>
<td>1 (2)</td>
</tr>
<tr>
<td>ras</td>
<td>WT1</td>
<td>1401-1402</td>
<td>24 (100)</td>
</tr>
<tr>
<td></td>
<td>WT2</td>
<td>1484</td>
<td>24 (100)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>A1401G/C-1402T</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MUT2</td>
<td>G1484F</td>
<td>0 (0)</td>
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<tr>
<td>codB</td>
<td>WT1</td>
<td>306</td>
<td>23 (43)</td>
</tr>
<tr>
<td></td>
<td>MUT1</td>
<td>M306V</td>
<td>5 (9)</td>
</tr>
<tr>
<td></td>
<td>MUT2</td>
<td>M306V</td>
<td>2 (3)</td>
</tr>
</tbody>
</table>

Values are numbers, with percentages in parentheses.

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Analysis of mutational pattern in multidrug-resistant tuberculosis (MDR TB) in a geographically...

Figure 1.
1a. Examples of GenoTypeMTDB probes strips (Hain Lifescience, Nehren, Germany). (Lane 1) Multidrug-resistant tuberculosis (MDR TB), rpoB S531L mutation and inhA C157 mutations. (Lane 2) MDR TB, rpoB S531L mutation and katG S315T1 mutation. (Lane 3) M tuberculosis, INH monoresistant (katG S315T1 mutation). (Lane 4) Mycobacterium tuberculosis, RIF monoresistant (rpoB S531L mutation in 530-533 region). (Lane 5) M. tuberculosis susceptible to isoniazid (INH) and rifampicin (RIF). (Lane 6) Negative control.

1b. Examples of GenoType M1DR probes (Lane 1) MDR TB with gyrA W1394N and embB M306V mutation (Lane 2) gyrA W1259P and embB M306I mutation (Lane 3) gyrA W1394N/V1394G and embB M306V mutation (Lane 4) MDR TB susceptible to Fluoquinolone, Cyclic peptides and Thiaminol (Lane 5) Negative control.
Comparison of drug susceptibility pattern of *Mycobacterium tuberculosis* assayed by MODS (Microscopic-observation drug-susceptibility) with that of PM (proportion method) from clinical isolates of North East India

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²(Department of Biotechnology, Assam University, Silchar)
³Hilaspur University, Hilaspur

ABSTRACT: About one third of the world's population are infected with tuberculosis and has become a serious global concern of public health. The major problem in controlling tuberculosis is the rapidity and efficacy of detection methods adopted. This study was conducted with the objective to compare the drug susceptibility pattern of *Mycobacterium tuberculosis* assayed by MODS (Microscopic-observation drug-susceptibility) with that of PM (proportion method) from clinical isolates of North East India. A total of 150 smear positive sputum specimens clinical isolates of *M. tuberculosis* referred to a clinical lab were selected. Both the proportion method and MODS were conducted for the collected isolates. DST of MODS were compared with that of PM considering PM as gold standard. MODS detected INH, RIF, STR and EMB resistant isolates at 31% (n = 46/150), 29% (n = 43/150), 19.3% (n = 29/150) and 27.3% (n = 41/150), respectively. Specificity was very high for all the drugs resistance with more than 99% specificity. The accuracy, PPV and NPV of MODS in detection of the four drug resistant isolates was relatively high. DST assay by MODS is relatively simpler. The appreciable performance characteristic in detecting drug resistance including MDR TB may lead to its wider applications in different labs and general hospital for resource limited regions.

KEYWORDS: drug susceptibility, MODS, PM, rapid detection, tuberculosis

1. INTRODUCTION

Tuberculosis arouses public health concern and became a global burden. It is about one third of the population of the world are infected with tuberculosis. AIDS and the increase incidence of multidrug resistant tuberculosis are the major factors that contribute to tuberculosis epidemic. In 2012, 8.6 million people fell ill with TB and 1.3 million died (WHO, 2013). TB occurs in every part of the world. In same year the largest number of new TB cases occurred in Asia, accounting for 60% of new cases globally. However, sub-Saharan Africa carried the greatest proportion of new cases per population with over 255 cases per 100,000 populations in 2012 (WHO, 2013).

In 2012, about 80% of reported TB cases occurred in 22 countries. Some countries are experiencing a major decline in cases, while cases are dropping very slowly in others (WHO, 2013). India is classified along with the sub-Saharan African countries to be among those with a high burden of tuberculosis as well as drug-resistant tuberculosis (WHO, 2012). Though India is the second-most populous country in the world, India has more new TB cases annually than any other country. In 2011, out of the estimated global annual incidence of 9 million TB cases, 2.3 million were estimated to have occurred in India (WHO, 2013). The nature and frequency of mutations in the resistant strains vary significantly based on the geographical location (Mokrousov et al., 2002). In this regard, here is very less information available on specific mutational patterns in India (Sharma et al., 2003), let alone on the underdeveloped and isolated region of northeast India. MDR TB strains have been reported mostly from countries where HIV and TB co-infection is endemic which includes India (Prasad, 2005).
The dual challenges of TB and HIV co-infections are particularly pressing in northeast India which has the highest average estimated adult HIV prevalence in India (NACO India, 2012). Undiagnosed and untreated cases continue to drive the epidemic in India. In 2010, an estimated 2.3 million TB cases occurred, and 360,000 patients died of TB, or about 1,000 deaths per day. Nearly one in six deaths among adults aged 15-49 are due to TB. Nearly 100,000 cases of serious multi-drug-resistant TB (MDR-TB) are estimated to occur in the country annually, and each MDR TB case costs more than 1 lakh to diagnose and treat (Singh et al., 2014).

The incidence/prevalence ratio in India is about 1:32. In case an efficient tuberculosis programme, targeting a sufficient number of sputum-positive prevalence cases in the community, is run for a sufficiently long period of time, it could bring down the prevalence, till probably the point when incidence and prevalence become equal in the community (NACO India, 2012). With an increase in the drug resistance of the Tubercle bacilli, the control of tuberculosis becomes more difficult.

In the present study comparative analysis has been carried out between conventional proportion method (PM) and Microscopic-obscuration drug-susceptibility (MODS) assay (New Rapid Susceptibility Test). This study also helps in increasing the understanding of TB occurrence and detection methods in NE India.

II. MATERIAL AND METHODS

2.1 Study settings

The clinical isolates referred to the Babina Diagnostic Centre in Imphal, Manipur were taken up for the present study. In order to strengthen the rapid detection of drug susceptibility of Mycobacterium tuberculosis at resources limited and high burden region the study was carried out.

2.2 Sputum specimens

One hundred and fifty sputum samples were collected for this study. Entire handling of the clinical specimen was performed inside a Class II safety cabinet in a BSL2 laboratory in accordance with CDC guidelines. Sputum decontaminations were carried out with the conventional N-acetyl-L-cysteine-NaOII. After centrifugation, the pellet was suspended in 2ml of 85% NaCl. All specimens were processed for acid-fast microscopy using Ziel-Nelsen technique (Canetti et al., 1993; Kent & Kubica, 1985). Sputum samples showing more than 10 acid-fast bacilli (AFB) per microscopic field in the smear were selected for the study.

2.3 Critical concentration of antibiotics in Drug Susceptibility Testing

The critical concentration of antibiotics which is given below Table 1 were maintained as per prescribed norms for indirect DST assay for PM and MODS methods.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Critical Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>0.2</td>
</tr>
<tr>
<td>Rifampicin (RMP)</td>
<td>40</td>
</tr>
<tr>
<td>Streptomycin (STM)</td>
<td>4</td>
</tr>
<tr>
<td>Ethambutol (EMB)</td>
<td>2</td>
</tr>
</tbody>
</table>

2.4 Proportion method

2.4.1 Culture preparation: Lowenstein - Jensen Medium is used with fresh egg and glycerol for the culture of Mycobacterium spp.
Comparison of drug susceptibility pattern...

2.4.2 Bacterial suspension for inoculation:
Approximately 1 mg moist weight of representative sample of the bacterial mass visualized as 2/3 loopful of 3mm internal diameter is added to 0.2ml of sterile distilled water in a 7ml Bijou bottle containing 10-12 glass beads. This mixture is vortexed for approximately 30 seconds to get uniform suspension. The suspension is then made up to approximately 1mg/ml concentration by adding more distilled water and then kept on the bench for 15-20min to allow coarser particles to settle down. From this suspension, a ten-fold serial dilution is made by adding 0.2ml to 1.8ml sterile distilled water. Each serial dilution suspension was inoculated by one standard loopful on to the drug-free as well as the drug-containing LJ slopes.

2.4.3 Interpretation of results:
The results are read for the first time on the 28th day. Colonies are counted only on slopes seeded with an inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculum ($10^7$ mg of bacilli) for the control slopes and the high inoculum ($10^8$ mg of bacilli) for the drug-containing slopes. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. Dividing the second figure by the first gives the proportion of resistant bacilli existing in the strain. Below a certain value — the critical proportion — the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages. If, according to the criteria indicated below, the result of the reading made on the 28th day is “resistant”, no further reading of the test for that drug is required and the strain is classified as resistant. If the result at the 28th day is “sensitive”, a second reading is made on the 42nd day and this provides the definitive result. Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs — rifampicin, isoniazid, ethambutol, and streptomycin — is classified as resistant to that drug. For calculating the proportion of resistant bacilli, the highest counts obtained on the drug-free and on the drug-containing medium should be taken, regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd.

2.5 Microscopic observation drug susceptibility assay (MODS)
The procedure was carried out as described by Moore et al (2006). MODS is conducted using Middlebrook 7H10 media. Culture preparation was done by dissolving 5.9g of 7H19 medium powder in 900 ml of sterile distilled water containing 3.1 ml of glycerol and 1.25g of casitone along with PANTA (polymyxin, amphotericin, nalidixic acid, trimethoprim, azlocillin) to minimize contamination of MODS culture by oral flora microorganisms not killed during decontamination process. Finally, 100μl INH 4μg/ml (Sigma) or 100μl RIF 10 μg/ml (Sigma) was added to the INH-containing well and RIF-containing well, respectively. The final concentrations of OADC (oleic acid dextrose catalase) and PANTA in each well were 10% and 20 μg/ml respectively. The drug concentrations in each well were maintained as per given in Table 1 and incubated at 37°C for 48 hr to verify sterility (lack of turbidity).

2.5.1 Bacterial suspension for inoculation:
Mix 10 ml sterile distilled water and 40 μl of 10% sterile Tween 80 in a sterile tube (final Tween 80 concentration = 0.04%). Using a sterile loop, harvest several colonies of Mycobacterium and place in a sterile tube containing 100 μl sterile distilled water-Tween 80 solution and sterile glass beads. Cap the tube tightly and vortex for 2-3 min (till there are no visible clumps). Let it stand for 5 min and the open tube and add 3 ml of water-Tween 80. Cap tightly and vortex again for 20s (till suspension has uniform turbidity). Let it stand for 30 min. Transfer the supernatant to another sterile tube using a pipette. Adjust turbidity to McFarland Scale 1 (approximately 3 x 10^6 CFU/ml) with 0.04% water-Tween 80 solution.

2.5.2 Plate setting for MODS:
A 24 well plate is taken and marked for controls and drugs-containing wells. 990μl of the culture media is dispensed on to the wells and the required drug concentration are added for drug containing well. The liquid inoculums are then added to make a final volume of 1ml. The plates are then closed with its lids and sealed with parafilm or with ziplock bags and incubated at 37°C (Sarman et al, 2012).
2.5.3 Interpretation of results:
The culture is determined to be susceptible when no growth is observed in both the wells. If one well in either the drug wells has \( \geq 2\text{cfu} \) growth while the other well has no growth or is intermediate (\( 0.5\text{cfu} \) to \( 2\text{cfu} \)) then the culture is termed as monoresistant to the drug with growth. If both the drug containing well of the culture has growth \( \geq 2\text{cfu} \) then it is considered resistant to both the drugs and is confirmed to be MDR. Fungal or bacterial growth indicates contamination. (Kent et al 1985; Caviedes et al 2002)

Table 2. Overall MODS culture interpretation.

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Overall culture interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined well findings (A&amp;B)</td>
<td>Positive</td>
</tr>
<tr>
<td>Both wells positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Both wells negative</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>Either well indeterminate</td>
<td>One well positive, other well negative</td>
</tr>
<tr>
<td>One well positive, other well indeterminate</td>
<td></td>
</tr>
<tr>
<td>Either well contaminated</td>
<td>Contaminated</td>
</tr>
</tbody>
</table>

Statistical methods were performed using SPSS 17.0. Results were considered significant at \( P \) value less than 0.05.

III. RESULTS

Table 3 below shows the result of DST to the four drugs for PM tested on 150 isolates. Of the total sample, isolates showing resistance to RH, INH, STY and KMR were 28\%(n = 42/150), 31.3\%(n = 47/150), 21\%(n = 31/150) and 21\%(n = 47/150) respectively by PM. Out of the 150 isolates, 26 were detected as MDR due to resistance to both INH and RH while 16 were monoresistant to RH and 21 were monoresistant to INH (Table 4). The results of the PM are taken as gold standard for comparison to that of MODS.

Table 3. Drug susceptibility pattern by proportion method

<table>
<thead>
<tr>
<th>NAME OF DRUGS</th>
<th>RESISTANT</th>
<th>SENSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIFAMPICIN</td>
<td>42</td>
<td>108</td>
</tr>
<tr>
<td>ISONIAZID</td>
<td>47</td>
<td>103</td>
</tr>
<tr>
<td>STREPTOMYCIN</td>
<td>31</td>
<td>119</td>
</tr>
<tr>
<td>ETHAMBUTOL</td>
<td>42</td>
<td>108</td>
</tr>
</tbody>
</table>

Table 4. MDR detection by proportion method

<table>
<thead>
<tr>
<th>Number of strains (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>susceptible</td>
</tr>
<tr>
<td>RH mono resistant</td>
</tr>
<tr>
<td>INH mono resistant</td>
</tr>
<tr>
<td>MDR</td>
</tr>
</tbody>
</table>
Comparison of drug susceptibility pattern...

Table 5. Drug susceptibility pattern by MODS method

<table>
<thead>
<tr>
<th>DRUGS</th>
<th>MODS</th>
<th>CONVENTIONAL PM</th>
<th>RESISTANT</th>
<th>SENSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RII</td>
<td>RESISTANT</td>
<td>42</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>RESISTANT</td>
<td>46</td>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>STR</td>
<td>RESISTANT</td>
<td>29</td>
<td>2</td>
<td>103</td>
</tr>
<tr>
<td>EMB</td>
<td>RESISTANT</td>
<td>41</td>
<td>1</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>SENSITIVE</td>
<td>0</td>
<td>0</td>
<td>108</td>
</tr>
</tbody>
</table>

Table 6. Performance of MODS in DST compared with proportion method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RII</th>
<th>INH</th>
<th>STR</th>
<th>EMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100(99 - 100)</td>
<td>97.9(95.6 - 100)</td>
<td>93.5(89.6 - 97.5)</td>
<td>97.6(95.2 - 100)</td>
</tr>
<tr>
<td>Specificity</td>
<td>99.1(97.3 - 100)</td>
<td>100(99 - 100)</td>
<td>100(99 - 100)</td>
<td>100(99 - 100)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.3(98 - 100)</td>
<td>99.3(98 - 100)</td>
<td>98.7(96.8 - 100)</td>
<td>99.3(98 - 100)</td>
</tr>
<tr>
<td>PPV</td>
<td>97.8(95.1 - 100)</td>
<td>100(99 - 100)</td>
<td>100(99 - 100)</td>
<td>100(99 - 100)</td>
</tr>
<tr>
<td>NPV</td>
<td>100(99 - 100)</td>
<td>99.0(97.5 - 100)</td>
<td>98.4(96.3 - 100)</td>
<td>99.1(97.6 - 100)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: NPV – negative predictive value, PPV – positive predictive value. Values are percentages with 95% confidence interval in parentheses.

Direct drug susceptibility testing results on MODS were compared with indirect DST on LJ as the gold standard for 150 samples. MODS detected INH, RII, STR and EMB resistant isolates at 31% (n = 46/150), 29% (n = 49/150), 19.3% (n = 41/150) and 27.3% (n = 41/150), respectively shown in Table 3. A comparison of the sensitivity of the four drugs in MODS shows a relatively low percentage in KTV (93.5%) with high value in RII (100%). Specificity was very high for all the drugs irrespective of more than 99% specificity. The accuracy, PPV and NPV of MODS in detection of the four drug resistant isolates given in Table 6.

IV. DISCUSSION

Our data shows that MODS is a sensitive and rapid method for diagnosis of TB and DST pattern. The sensitivities in detection of INH and RII resistance in our study were lower than those from the study of Moore et al 2000 (72.6% vs 84.6% for INH and 72.7% vs 100% for RII) although both studies used the same INH concentration (0.4 μg/ml) and RIF concentration (1 μg/ml). These concentrations have been recommended in the MODS guidelines from the MODS development team in Peru (Jorge et al; 2009). However, a recent meta-analysis published after completion of this study concluded that the sensitivity of INH-resistance detection was higher with a concentration of 0.1 μg/ml without loss of specificity (Minion et al., 2010). The only equipment needed to perform the MODS assay is an inverted microscope, tissue culture plate and consumables, biological safety cabinets and incubator. The technical competence required is aseptic technique and microscopy skills. A commercial MODS plate (TB MODS kit™) has been developed by Hardy Diagnostics, USA in collaboration with PATH and is under evaluation.

V. CONCLUSION

In conclusion MODS is appropriate for screening for DST pattern in high burden countries where such tests are urgently needed. MODS meets many criteria for an DST diagnostic test applicable for high-burden settings; it is rapid, low cost and accurate and can be performed without the need for biological safety level 3 laboratories (if the plate is not opened after inoculation). Therefore, MODS is an alternative method for rapid DST screening in these settings. Recently, wide application of MODS in resource-constrained settings has been endorsed by WHO (2011). However, an international standard operating procedure and a quality assurance system accredited by WHO should be developed to standardize and maintain accuracy.
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