Chapter 5
DISCUSSION

The demographic detail shows 20% of children with TB infection in our study, which is quite lower as compared with 37.7% children infection detected by study of Nelliyanil et al. (2012). The same study found only 23% of relapsed cases of TB which is quite low as compared with our study finding with 47.5% indicating lack of proper knowledge about proper intake of drugs prescribed by physician or it may be due to higher percentage of HIV infection in Northeast region or it may be due lack of awareness of TB in the region.

MDR detection by proportion method in our study is quite low as compared with the study carried out by Acharya et al. (2008) (12.5% vs. 29.9%). The same concentration of drugs were used in both studies thus the variation in MDR detection may be due to lesser prevalence of MDR in our study region as compared with the study region (Nepal) carried out by above mentioned author or it may be due to larger sample size in the authors’ study which is quite probable. Same author detected 28.5% culture negative while our study showed only 4% culture negative confirmed by Bact/Alert 3D method.

Our data shows that MODS (microscopic observation drug susceptibility) is a sensitive and rapid method for diagnosis of TB and MDR-TB. The sensitivities in detection of INH (isoniazid) in our study was higher than the study of Moore et al. (2006) (97.9 % vs. 84.6%) but same sensitivity of 100% was seen for RIH (rifampicin) resistance in our study and those from the study of Moore et al. (2006). Although both studies used the same INH concentration (0.4μg/ml) and RIH concentration (1 μg/ml). These concentrations have been recommended in the MODS guidelines from the MODS development team in Peru (Jorge et al., 2008). 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 are an inverted microscope, tissue culture plate and consumables, biological safety cabinet 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. MODS is appropriate for screening for MDRTB in high burden countries where such tests are urgently needed. MODS meets many criteria for an MDR TB 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 MDR-TB 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.

Higher accuracy results for INH, RIH and STR and lower accuracy results for EMB were shown in a similar multicentre study (Martin et al., 2005). However, marginally lower sensitivity and specificity for STR and EMB have also been reported (Montoro et al., 2005 and Lemus et al., 2006). For STR and EMB testing, performance differences were seen among different laboratories rather than drug sensitivity testing method which is argued as inherent difficulties of testing with these two drugs stressing the need for continuing participation in proficiency testing (Laszlo et al., 2002). LJ or agar medium is time consuming.

For developing countries, it would be helpful to have a simple and inexpensive test that can rapidly detect resistant *M. tuberculosis* strains. The reporting time of NRA was between 7 - 14 days with majority in 10 days as against 28 - 42 days for the conventional PM (proportion method). This is very much comparable with the results of MGIT and BACTEC 460 TB, which require the use of expensive instruments and high running cost. The NRA utilizes standard solid LJ-medium, although with KNO₃ incorporated and it could therefore be easily adopted in any culture laboratory. False susceptible results would in this case be detected by the lack of a positive reaction also in drug free growth. *M. bovis* does not reduce nitrate, therefore the NRA technique is not applicable. Disadvantages of NRA are; the culture is killed by the mix reagent used to develop the assay, requiring that multiple cultures be prepared if comparative testing will be performed and only fresh cultures must be used (<14 days).
Reports on drug susceptibility testing by NRA are generally favourable for INH and RIH (Cohn et al., 2004; Sethi et al., 2004; Huberto et al., 2004; Somoskovi et al., 2001). Noting that both drugs are major first-line anti-tuberculosis agents and considering that rifampicin is a surrogate marker for MDR- M. tuberculosis (Rattan et al., 1999; Somoskovi et al., 2001), the NRA could be used to screen for resistance to both drugs to enable prompt assessment of MDR prevalence particularly in highly endemic regions. The relatively low sensitivity rates of STR observed in this study require further evaluation. Previous studies reporting similar findings have suggested a review of the drug concentrations (Cohn et al., 2004; Sethi et al., 2004; Huberto et al., 2004). Considering that streptomycin is generally used for the treatment of other clinical conditions and therefore subject to abuse, the NRA needs to be adequately controlled and doubtful results confirmed by a gold standard method.

In the study of Nair et al. (2009), the complete agreement of 100% between Bact/Alert 3D and proportion method was shown for drug susceptibility test but in our study there is quite difference in the drug susceptibility test between the two methods despite using same concentration of drugs. Although the time of detection of drug susceptibility results by Bact/Alert3D has complete agreement with our study. The MDR detected was of 16% (n=60/375) in our study which is in complete agreement with the study of Hemrani et al. (2002) and little higher than study of Nair et al. (2009) with 15.7% MDR detected by Bact/Alert 3D. Similar levels of MDR TB have been reported by various workers from different parts of the country and a higher level was reported by Malhotra et al. (2002) (24.3%).

Proportion method could detect only 47 MDR strain while Bact/Alert 3D detected a total of 60 MDR strains proving its advancement from the conventional proportion method which is quite reasonable. Further advancement in comparative studies is needed in order to confirm the most efficient method among the two methods mentioned here. The colorimetric method using the BacT Alert 3D system is relatively new. This system could perform the susceptibility testing in 8 days compared to 20 days by the LJ-method (Nair et al., 2000). There was a 40% reduction in time using the automated machine. The drug resistant isolates took ≥10-12 days by automation and 27-32 days by LJ method to grow which, was longer than the sensitive isolates. The drug resistant isolates took longer to grow compared to the sensitive strains by automation; a similar finding has been reported by Toungoussova
et al. (2005). The time taken to perform susceptibility was well within the period of 4 weeks delineated by Tenover et al. (1993).

The efficiency of Genotype MTBDR have been reported by many authors (Hilleman et al., 2006, 2007 and 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 and Tessema et al., 2012). This may be due to specific location independently developing endemic mutations. The present study evaluates the INH, RIH, MDR and EMB mutations using genotypic MTBDRplus and MTBDRsl test compared with BacT/Alert 3D DST of a geographically isolated region of northeast India.

The performance characteristics of specificity, sensitivity, accuracy, PPV and NPV as given in Table 24 suggest that the MTBDRplus assay is equivalent to Bact/Alert3D culture based drug susceptibility test performed in quality assured reference laboratories but detection of ethambutol resistance by MTBDRsl, though lower than INH or RIH detection by MTBDRplus, is comparatively higher than other reported studies (Brossier et al., 2010 and Tessema 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 ndh and kasA gene (Lee et al., 1999, 2001 and Rindi et al., 2005). Three out of 16 INH mono-resistant strains were detected by mutations in inhA gene only. These mutations would not have been detected by the previous MTBDR which did not 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 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 (Foundation for Innovative New Diagnostics)-negotiated prices, the cost of molecular assay is less than 50% of that for conventional liquid culture and DST for INH and RIH (Barnard et al., 2008). Therefore there is urgency for an efficient method of TB testing as a complement to conventional culture and smear microscopy.

The results of the study showed that the mutational pattern of the resistant genes did not 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 RIH resistance is the Ser-531-Leu mutation (45.6%) and in INH resistance, the Ser-315-Thr1 is the most common (73%). This is more or less in agreement with earlier reported studies (Miotto et al., 2006 and Mokrousov et al., 2002). In case of EMB resistance, Met-306-Val mutation (46.5%) is observed to be the major cause. Previous studies have shown that 40-95% of isoniazid resistance are defined as the high level drug resistance due to katG gene mutations. In this present study, 87.5% of INH mono-resistant are attributed to katG mutations of which 81.3% are S315T1 and 6.3% 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 on-going 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 Genotype MTBDRs/l method in our study detected a MDR positive sample having rare positive band at the mutation region of D94H. It is worth noting that D94H is described in the MTBDRs/l instruction manual as a mutation predicted from in silico studies and not previously detected in vitro, a prediction that is now confirmed in vivo (Brossier et al., 2010). The author also detected same rare mutation in a single strain, which is in agreement with our study proving the efficiency of MTBDRs/l method in detecting rare mutation of fluoroquinolone drug at gyrA gene region. The most common mutation in our study in gyrA gene was seen in the D94G region as seen in the study of Ajbani et al. (2012). However, the percentage of mutation observed in the study was 17.2% (n=10/58) whereas it was 42.26% in the study of Ajbani et al. (2012). This variation is quite probable as variation in the sample size and the differential geographical location cannot be rule out too.
ARDRA was found to be a useful tool for identification of mycobacterial isolates in a clinical routine laboratory, because of its speed compared to phenotypic identification, its reliability, practical applicability, flexibility and the possibility to identify most non-tuberculous mycobacteria together with and at the same cost as \textit{M. tuberculosis}, at an affordable price. Several studies based on restriction digestion of the amplified 16S rRNA gene (Hughes \textit{et al.}, 1993; Dobner \textit{et al.}, 1996; Jeng \textit{et al.}, 2001 and Baere \textit{et al.}, 2002) and the 16S–23S rRNA gene spacer (Roth \textit{et al.}, 2000) have been reported. The target of the current ARDRA fingerprinting was part of the 16S and the 23S rDNA regions as well as the 16S–23S rDNA gene spacer. In our study the 16S rRNA was utilised and found significant importance in differentiation of mycobacterial \textit{spp.} among the clinical isolates as observed in previous studies by different authors.

The inclusion of the spacer region is advantageous because this region is less conserved than the 16S rDNA and shows considerable sequence and length polymorphism at both the genus and species level (Abed \textit{et al.}, 1995) but in our study the polymorphism within the \textit{Mycobacterial tuberculosis} complex could not be seen and it is in agreement with previous studies by Liebana \textit{et al.} (1996); Glennon \textit{et al.} (1994) and Niemann \textit{et al.} (2000).

Technically, ARDRA is non-demanding, comprising only basic molecular biology techniques like simple DNA extraction, PCR, restriction digestion and submarine agarose gel electrophoresis (Baere \textit{et al.}, 2002). The four species of the \textit{M. tuberculosis} complex showed identical fragment patterns suggesting that ARDRA may not be suitable for intra complex differentiation. Previous studies have documented that species within the \textit{M. tuberculosis} complex share large sections of their genomes such as the 16S rRNA genes, the 16S–23S internal transcribed sequences, and specific repetitive elements such as the direct repeat locus (Liebana \textit{et al.}, 1996; Glennon \textit{et al.}, 1994 and Niemann \textit{et al.}, 2000) and the similar result is seen our study. The species of the \textit{M. tuberculosis} complex cannot be differentiated on the basis of the 16S rDNA sequence, and therefore restriction digestion of this gene could not either. Most of the clinically relevant and the most abundant species were readily differentiated from each other.
Mycobacterium spp. were identified by a direct sequence determination of amplified 16S rRNA gene fragments in our study as studied by Kirschner et al. (1993) and Springer et al. (1996). The 16S rRNA sequence analysis in our study suggested a way to identify mycobacteria spp. by characterizing species-specific nucleotide sequences as studied by Bodinghaus et al. (1990); Clayton et al. (1995) and Rogall et al. (1990). As the 16S rRNA sequencing cannot differentiate Mycobacterial tuberculosis strains within the spp. a deep study in this region of gene for resolving the intra-species of Mycobacterial tuberculosis is indicated by our findings. Overall we find sequencing technology to be an excellent tool for species identification of mycobacteria.