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Analysis of one round of annual proficiency testing results of Intermediate reference laboratories conducted by the National reference laboratory.

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Abstract
Objectives: The annual proficiency testing of state level laboratories is conducted by the National Reference Laboratory (NRL) to assess the efficiency of the laboratories to correctly identify Mycobacterium tuberculosis and to determine its drug susceptibility pattern. The proficiency testing results of the year 2016 were analyzed to identify problems and provide corrective measures to overcome technical difficulties in reporting accurate results.
Methods: A panel of twenty cultures were sent out to each of the 15 laboratories certified for solid culture liquid culture and Line probe assay. The laboratories were expected to grow the cultures in their own laboratories, set up drug susceptibly testing by all the methods they were certified for and report the results within the stipulated time period.The turnaround time for reporting results, specificity, sensitivity positive and negative predictive values and efficiency of the laboratory in identifying the cultures were analyzed.
Results: Most of the laboratories had reported their results within the stipulated time period. Only 40% of the laboratories had 100% efficiency in solid cultures while 90% of laboratories had achieved 100% in the liquid culture method. Almost all laboratories had achieved 100% efficiency in the line probe assay method.
Conclusion: Since the liquid culture and line probe assay technologies are routinely used for the detection of drug resistant tuberculosis the laboratories exhibited higher level of efficiency as compared to solid culture & drug susceptibility testing which are rarely used.

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I. Introduction:

The Global report 2016 had reported that India has the highest burden of Tuberculosis (TB) and multi drug resistant (MDR) TB. An estimated 1.3 lakh incident multi-drug resistant TB patients emerge annually in India.[1]To combat this menace good quality assured culture and Drug susceptibility testing laboratories are required for the diagnosis of MDRTB. In 2007, the Revised National Tuberculosis Control Program (RNTCP) rolled out DOTS Plus services for the management of MDRTB in two states *ie* Gujarat and Maharashtra.[2] Two intermediate reference laboratories (IRL) had been established in these two states to perform culture and drug susceptibility testing (DST) for diagnosing DRTB. Subsequently, the program has been successful in establishing at least one Intermediate Reference Laboratory (IRL) in each state. By 2016, the program had established and certified 45 solid culture &DST, 51 LPA and 28 liquid culture laboratories.[3]Theselaboratories had to be quality tested periodically, to ensure accurate reporting of DST results. For this purpose the National Reference Laboratories (NRL's) were given the charge of conducting annual proficiency testing for these state level laboratories. Currently, these state level laboratories are supervised and monitored by six NRL's. The NRL's are responsible for capacity building of the state level laboratories, provide technical inputs and ensure quality diagnosis. This is achieved by periodically organizing training programs at the NRL as well as on site, undertaking periodic on site evaluation visits and by conducting external quality assurance program for sputum smear microscopy & culture and DST.

II. Material And Methods

A panel of twenty cultures were sent to the certified laboratories in Karnataka, Maharashtra and Rajasthan in the month of May 2016. The panel consisted of cultures with different drug susceptibility testing pattern. The laboratories were required to grow these cultures and perform drug susceptibility testing by the

method that they were certified for by RNTCP. The laboratories had to follow the standard operating procedures present in the national guidelines.

Solid culture: As per the RNTCP guidelines,[4] solid culture and DST had to be performed on Lowenstein Jensen Medium. The LJ medium was prepared in house by blending country eggs with mineral salt solution. 2% malachite green had to be incorporated in to the medium to control contamination. The drug susceptibility testing of isolates was performed by the proportion sensitivity test. Isolates were tested for first line anti TB drugs with the critical concentration of 4µgm/ml for Streptomycin, 0.2µgm/ml for Isoniazid, 40µgm/ml for Rifampicin and 2µgm/ml for Ethambutol.

Liquid culture: The MGIT 960 liquid culture system is used in all the laboratories. The BBL™ MGIT™ 7 mL Mycobacteria Growth Indicator Tube is a tube containing a modified Middlebrook 7H9 Broth which supports the growth and detection of mycobacteria. The MGIT tube is a 16 x 100 mm round-bottom tube which contains a fluorescent compound embedded in silicone on the bottom of tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The initial concentration of dissolved oxygen quenches the emission from the compound, and little fluorescence can be detected. Later, actively growing and respiring microorganisms consume the oxygen which allows the compound to fluoresce. The BACTEC MGIT 960 SIRE Kit is a 4 – 13 day qualitative test. The test is based on growth of the *M.tuberculosis* organism in a drug-containing tube compared to a drug-free tube (Growth Control). The BACTEC MGIT 960 instrument continuously monitors tubes for increased fluorescence. Detection of fluorescence in the drug-containing tube is compared with the fluorescence in the Growth Control tube. This is utilized by the instrument to determine susceptibility results. The BACTEC MGIT 960 instrument automatically interprets these results and reports a susceptible or resistant result. First line DST was set up using the SIRE kit supplied by the manufacturer (Becton Dickenson). The critical concentration of the drugs were 1.0 µg/ml for Streptomycin, 0.1 µg/ml for Isoniazid, 1.0 µg/ml for Rifampicin and 5.0 µg/ml for Ethambutol.[5]

Line Probe assay: The Genotype MTBDR plus test is based on the DNA STRIP technology and permits the molecular genetic identification of the *M.tuberculosis* complex and its resistance to rifampicin or isoniazid from culture isolates or directly from smear positive pulmonary sputum samples. Rifampicin resistance is detected by the presence ofmutations of the rpo B gene (coding for the beta sub unit of the RNA polymerase). For testing high level of isoniazid resistance the kat G gene (coding for the catalase peroxidase) is examined and for testing low level isoniazid resistance, the promoter region of the inh A gene (coding for the NADH enoyl ACP reductase) is examined.Line probe assay technology involves 3 steps.

1. Extraction : DNA is extracted from M.tuberculosis isolates or directly from clinical specimens.
2. Amplification: Amplification of the resistance-determining region of the gene under question is performed using biotinylated primers by the polymerase chain reaction (PCR).
3. Hybridization : labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of M. tuberculosis complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations.
4. Interpretation : The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding and is observed by eye.

1.5ml of each isolate were sent in cryovials to each of the participating laboratories. The cultures were packaged in triple layered packing and were dispatched to laboratories via speed post. They reached the laboratories within one or two days. The laboratories intimated the NRL upon receipt of the strain, the time to detection was calculated from the day the strains were received at the laboratory.

The NRL used a 'judicial result' as a proxy gold standard for evaluation of the results. A judicial result was available if more than 90% of all valid results reported were concordant. Below that level, a judicial result was unavailable and the strain for that drug was excluded from the analysis. The judicial resultwas used as the gold standard to determine sensitivity, specificity, and efficiency. Sensitivity, specificity and efficiency were defined as the proportion detected of the strains judicially classified as resistant, susceptible or both.

III. Results

The results were first compiled and their specificity sensitivity, positive & negative predictive value and efficiency were calculated. The number of laboratories that reported for each of the DST method is presented in Table.1.

The performance of different laboratories in terms of specificity, sensitivity, positive & negative predictive values and efficiency for first line drugs on solid culture and liquid culture & DST were analyzed and are given in Figure.1 and Figure.2.

16 laboratories participated in the proficiency testing for LPA and all of them obtained 100% specificity, sensitivity, Positive and Negative predictive value and efficiency.

The turn around time to detection was also analyzed for the various culture methods. The turn around time for solid and liquid culture is presented in Figures.3 and 4 respectively.

IV. Discussion:

Continuous monitoring and evaluation of laboratories performance is important for reporting quality results. A laboratory quality assurance program is hence very essential to ensure no false results are declared. There are many agencies conducting the quality assurance programs. This involves sending out a panel of test organisms and asking the laboratories to correctly identify the microorganism. Usually participating laboratories are required to pay these agencies for the testing services they render. For the RNTCP certified laboratories, it was decided by the National Laboratory coordination committee that the National reference laboratories (NRL) would be responsible for conducting the quality assurance program. The NRL's would send out a panel consisting of 20 cultures to all the certified laboratories supervised by that particular NRL. The technologies that the laboratory would be tested are those which are routinely used for patient diagnosis. The expected efficiency for Isoniazid and Rifampicin was more than 90% while it was 85% for Streptomycin and Ethambutol. For the second line drugs, the efficiency was expected to be more than 90% for all the drugs. [6]

With the introduction of rapid molecular diagnostic tools such as LPA, solid culture &DST results is not used for patient diagnosis. Solid culture and DST is technically very demanding with regards, to preparation of inoculum, preparation of drug containing LJ medium and inoculation of media. If not routinely done, the technician performing the test may lose his or her proficiency. This may result in erroneous results.This scenario is clearly evident in the present analysis undertaken by the NRL. The efficiency of all laboratories in performing LPA was 100% whearas the same laboratories did not score 100% for solid culture & DST. This was expected as LPA is routinely used for MDRTB diagnosis and not the conventional culture system. However, the laboratories had performed well in liquid culture and DST. This may be due to the fact the liquid culture labs were newly established and have recently been certified. Specifically, laboratory number 11 had performed poorly for Streptomycin on solid culture with an efficiency of 74%, while the same laboratory had achieved 100% for Streptomycin on liquid culture. The laboratory had mostly reported false resistant results in solid DST. This may be due to lower drug concentrations in the medium. Another laboratory which is certified only for solid culture had an efficiency of only 69% for Streptomycin. The laboratory had reported 7 false resistant results. This again may be due to problems with media preparation, which may not contain less drug. Considering the results from two laboratories it appears that the main reason for lowered efficiency in Streptomycin drug solid DST appears to be the concentration of the drug in the medium. The turn around times for detection also were analyzed. Lab number 11 had taken 253 days to report liquid culture and DST results. This delay was due to non functioning of their BSL3 facility. Another lab had taken 161 days to report liquid culture & DST results. The reason for this delay was due to problems with the availability of trained staff and routine workload of the laboratory.

It appears that for timely and accurate reporting of results, proper functional infrastructure of the laboratory, functional BSL3 lab, trained staff and appropriate management of sample inflow & work distribution among staff is crucial for delivering accurate results.[7,8,9]Apart from these the proficiency of trained staff seems to deteriorate over time when they donot routinely use a particular technique. To maintain the proficiency in a particular technique, the laboratory head may take it upon himself to periodically use the technology which is less often used.

We wish to conclude that the quality assurance program conducted by the NRL should continue and include newer labs that are certified.

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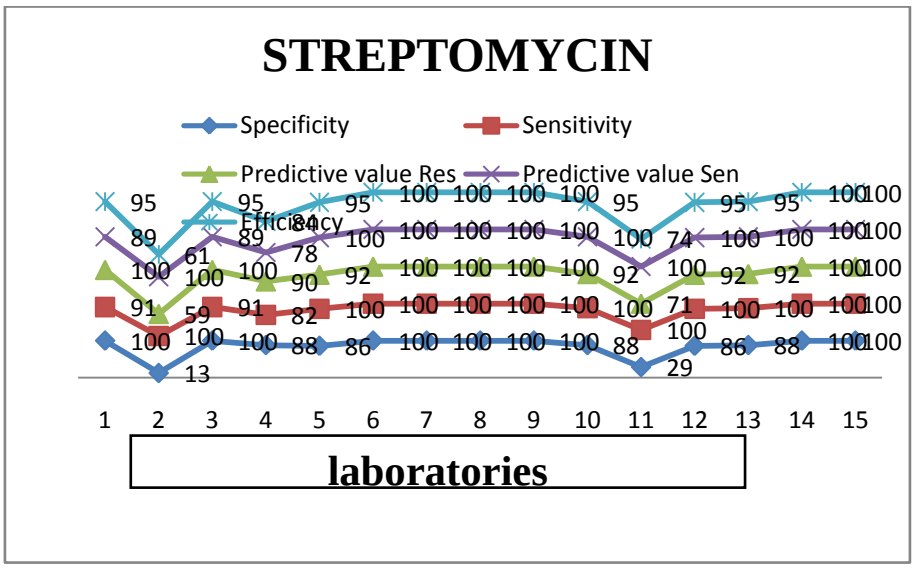
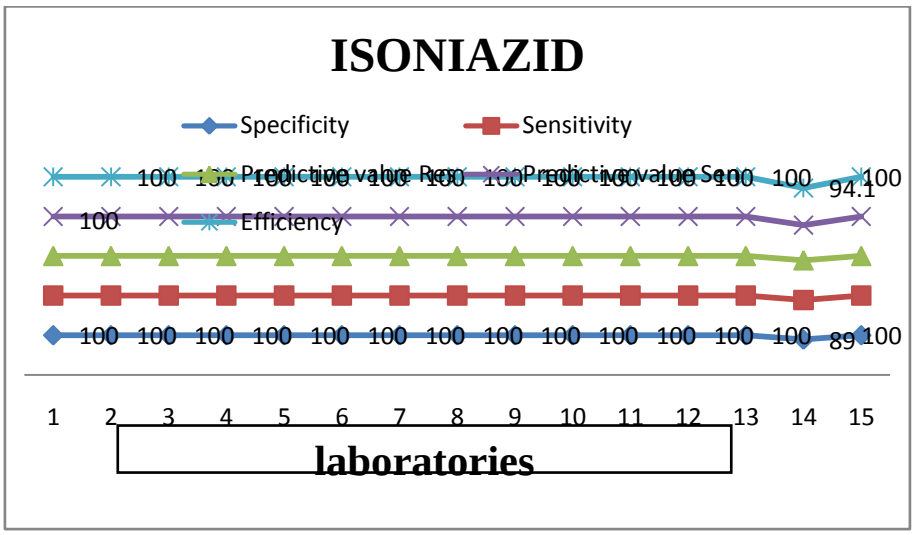
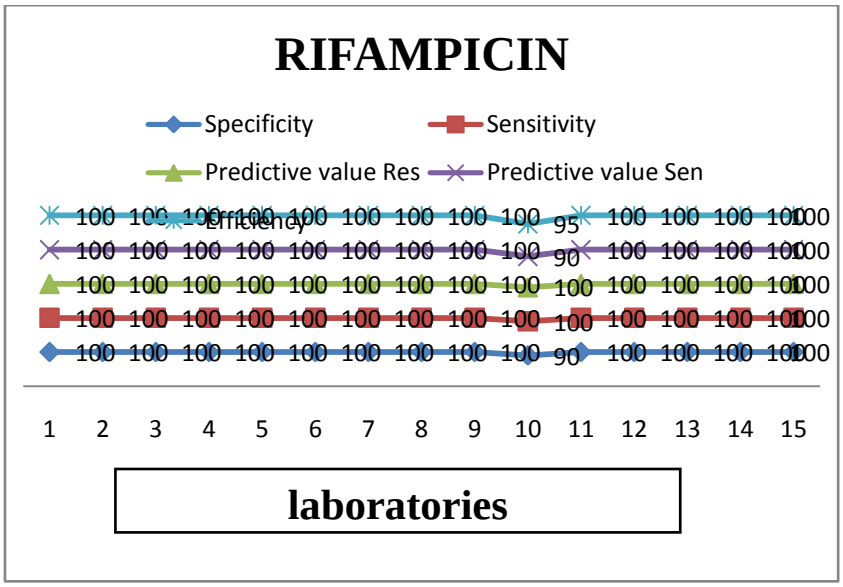
FIGURES

Figure1. Performance of laboratories in solid culture & drug susceptibility testing for Streptomycin, Isoniazid, Rifampicin and Ethambutol.







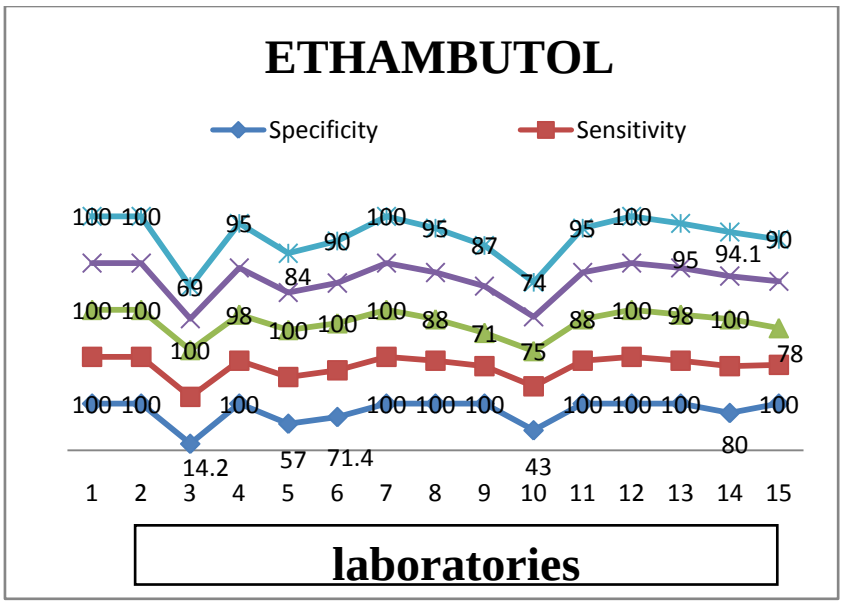
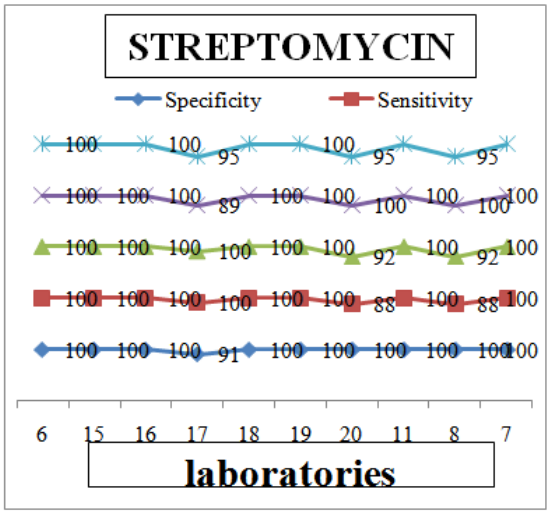
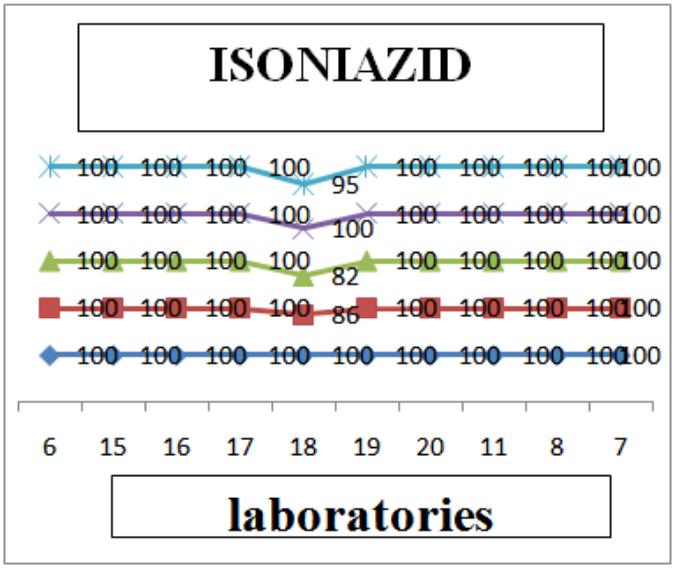


Figure 2. Performance of laboratories in liquid culture & drug susceptibility testing for Streptomycin, Isoniazid, Rifampicin and Ethambutol.

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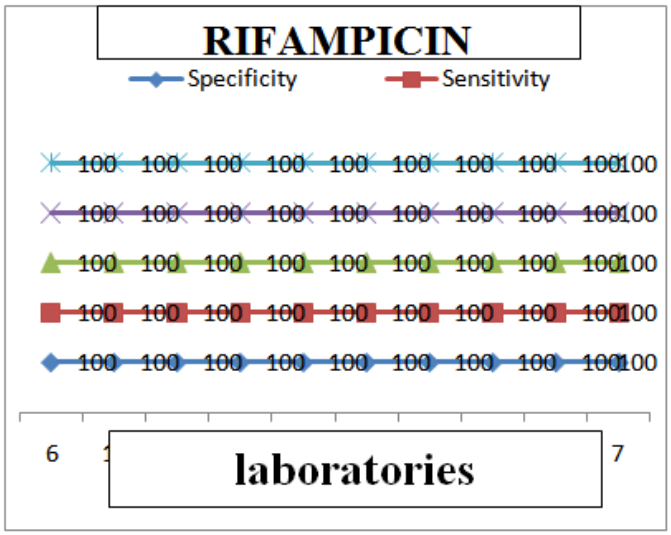
laboratories


RIFAMPICIN
Specificity
Sensitivity
6
7
laboratories

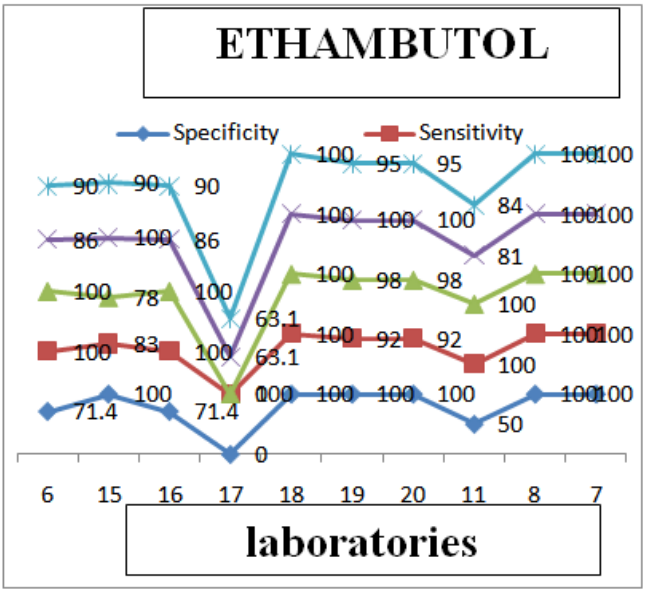

ETHAMBUTOL
Specificity
Sensitivity
6 15 16 17 18 19 20 11 8 7
laboratories

Figure.3 turnaround time of laboratories for reporting solid culture & drug susceptibility testing results

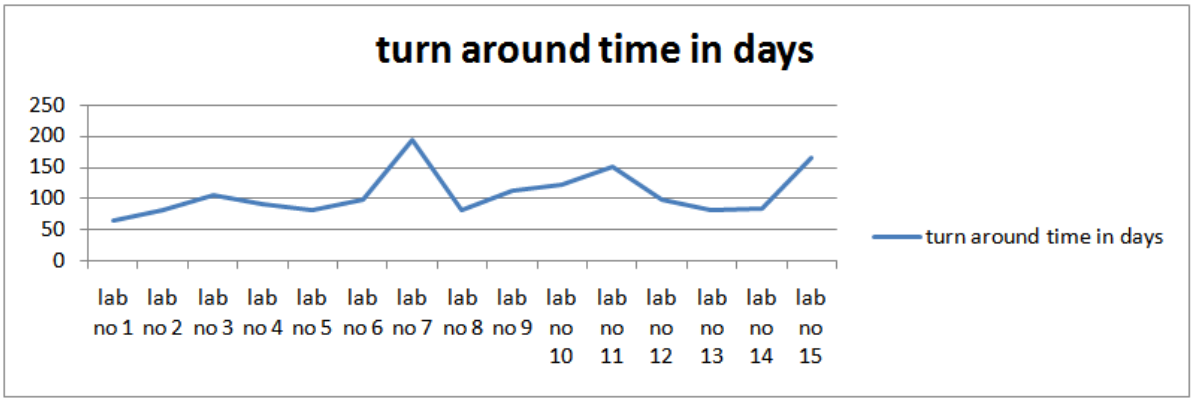


Figure.4 turnaround time of laboratories for reporting liquid culture &drug susceptibility testing results

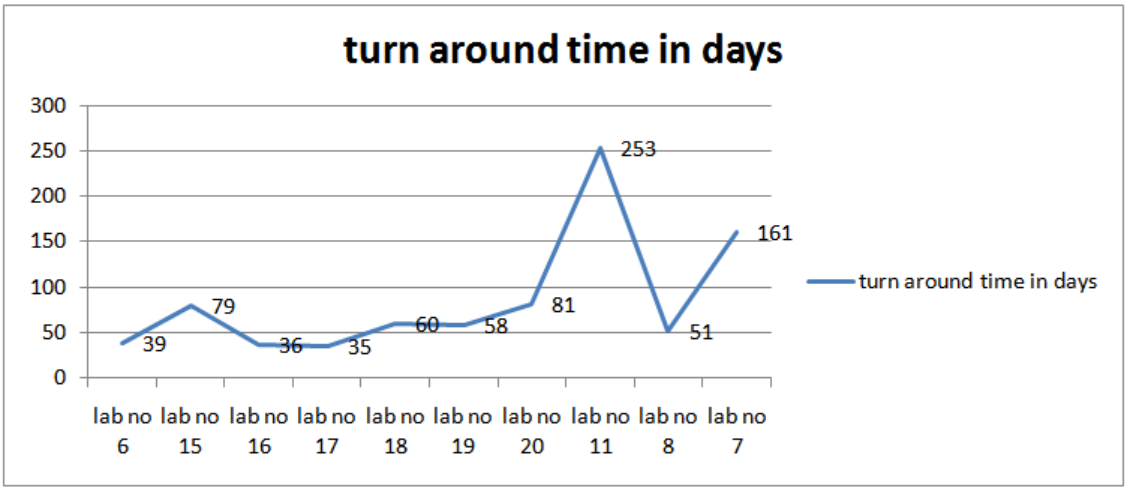


Table.1 Number of laboratories that were tested for each of the testing modalities.

Method	Number of laboratories
Solid culture Proportion sensitivity testing	15
Liquid culture First line by MGIT	6
Line prone assay	16

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