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An interdisciplinary parallel methodological approach for the diagnosis of tuberculosis in the Central state of India.

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Abstract: *Early and accurate diagnosis of tuberculosis is extremely important for its control. The classical staining and microbiological methods lack in accuracy and speed respectively. On the other hand, the accurate and faster advanced nucleic acid amplification (NAA) methods have their own limitations and the results need to be interpreted with much caution for therapy. To balance the requirements of accuracy and speed for aiding early and correct drug prescription to the patients, we used a combined classical and molecular biological approach.*

Using this approach, 244 pulmonary and extra-pulmonary clinical samples from tuberculosis suspects from the Central state of India were tested for the presence of M. tuberculosis. Of these, 84 samples were finally confirmed positive for M. tuberculosis. Most positive samples were from clinical suspects in the age group of 20-24 and 35-39. Analysis of the positive samples showed that the peak at 20-24 was dominated by male while 35-39 was dominated by female gender. We also found gender based difference in the proportion of some of the different type of positive samples. We present here, the status of tuberculosis infection in Central state of India in 2012-2014 tested in our lab.

Keywords: *conventional and advanced molecular methods, diagnosis, Mycobacterium tuberculosis*

I. Introduction

Tuberculosis (TB) is a chronic infectious disease, having high morbidity and serious health implications for those infected [1]. TB is caused by the Mycobacterium tuberculosis complex (MTC), which includes *M. tuberculosis, M. bovis, M. africanum, and M. microti*. TB is initially asymptomatic, until the bacteria reach its active stage. It is mainly a pulmonary disease, but can also spread and disseminate into the bloodstream to infect extra-pulmonary organs[1]. The occurrence of the disease is as high as one in five registered TB patients [2–4]. The disease occurs in people with impaired immunity and commonly co-occurs among patients with HIV infection. In women, TB is especially associated during pregnancy and often leads to maternal mortality.

Indian Council of Medical Research studied the prevalence of TB in India and established the 'Directly Observed Therapy Short course' (DOTS) strategy approved by the World Health Organization (WHO) and the Revised National TB Control Programme (RNTCP) [5]. Since 2012, RNTCP declared TB as a 'notifiable disease of the nation' [6]. Despite a 45% decline in global TB mortality rates and a slow decline of the global incidence of TB, the burden of TB is still large and it remains one of the deadliest diseases of developing countries[1]. Annually, India has more new cases of TB than any other country [6] and in 2012 India accounted for 26% of incident cases [1]. Of the 8.6 million incident TB cases in 2012, 34% were women with South-East Asian region accounting for 68% cases among women [1].

Although TB and its symptoms were identified a long time ago; accurate diagnosis of the disease is still a challenge. Symptoms differ in patients and are associated with development of new resistant strains. The increasing numbers of new TB cases and the need to provide early and effective drugs to the infected patients necessitates rapid and specific diagnostic assays for *M. tuberculosis*. Initially, the laboratory procedure for diagnosis included the Mantoux tuberculin skin test approved by the Center for Disease Control and Prevention (CDC). New methods have since been developed, improving the diagnosis with more sophisticated machinery and histopathology, microbiology and molecular biology methods [7–9]. Classical methods of TB diagnosis like staining of the acid fast bacteria (AFB) may give large false negative results while the "gold standard" detection of tubercle bacilli through bacterial culture is rather slow. Most of the nucleic acid amplification (NAA) assays are both rapid and specific to the tubercle bacilli. Despite these methods falling short of their theoretical ability to detect even a single mycobacterium cell, NAA tests are still more reliable [10] and are expected to totally replace the conventional diagnostic methods for detecting TB. However, these tests are not free from false negative results and the results need to be interpreted cautiously [11].

Treatment of tuberculosis is also still challenging. Prescription of correct drugs depends upon the methodologies for diagnosis of tubercle bacilli after medical patho-physiological analysis of the disease. Such analyses include

X-rays, sonography, multi detector computed tomography (CT), and Magnetic resonance imaging (MRI)[12,13]. The current control relies heavily on detecting infectious cases and treating them for at least six months with a combination of antibiotics – a therapy aimed at curing the patients and achieving non-infectiousness. There is still no completely effective vaccine to control TB which makes it extremely important to interrupt the transmission of TB through early, accurate diagnosis and targeted treatment to prevent the emergence of further drug resistance in the tuberculosis bacteria.

In the present study on TB diagnosis, we utilized a strategic combination of methodologies for identification of mycobacterial infection to overcome the shortfalls of each of these methods. The methods included histo-pathological study, microbiological methods – which allow visualization of infectious bacteria by microscopy after AFB staining, followed by the culturing of patient's samples – along with a molecular biological approach to specifically identify M. tuberculosis. We tested multiple types of samples from patients, provided by medical specialists from different hospitals, for the diagnosis of pulmonary and extra-pulmonary tuberculosis. Apart from the usual samples for diagnosis of extra-pulmonary TB, we also analyzed semen and menstrual blood samples from patients suspected to have genito-urinary TB as genito-unrinary TB is one of the most common late manifestation of earlier symptomatic or asymptomatic pulmonary TB and is responsible for 30-40% of extra-pulmonary cases[14].

Specialists in the different fields of microbiology, histo-pathology and molecular biology performed the tests and analyzed the data obtained using these different methods. Finally, the infecting organism was identified at species level using molecular biology tools. The organism was then tested for sensitivity against first and second line drugs followed by prescription recommendations. In case the organism was found to be Non-tubercle mycobacteria (NTM), drugs against these were recommended. Our work is thus an example of diagnosis improvement using a strategically combinatorial method leading to a more personalized and directed treatment of TB. We further analyzed our data to check for correlation between age and gender of the patient with the positive diagnosis for TB.

A few studies do compare conventional and advanced molecular methods for the diagnosis of TB[15,16]. A study on diagnosis of pulmonary and extra-pulmonary tuberculosis from Central India using bacteriological methods was reported earlier [17]. To our knowledge, ours is the first study that uses a combinatorial approach for TB diagnosis.

II. Subjects and Methods

2.1. Population and samples

A total of 244 target samples were obtained from clinical TB suspects from the Central state (Madhya Pradesh) of India to be used for this study. These samples were obtained and diagnosed with the support of professional medical specialists at hospitals like CHL and Rajshree hospitals in Indore, Ujjain and Jabalpur districts of the Central state of India. Specifically, patients who were suspected by the medical specialists for pulmonary or extra pulmonary TB through clinical testing were selected. Samples were obtained depending on the type of TB suspected. Patient demographic data like age, gender and address were also obtained. Samples obtained from the hospitals were transported immediately to the laboratory under cold temperature conditions for preservation. The samples were processed on the same day or were kept at $+4^{o}C$ until they were processed.

2.2. Strategy for use of the combinatorial parallel use methodology for TB diagnosis

Classical and modern molecular methods were used together for obtaining more accurate TB diagnosis. This included microbiological smear and culture methods, histo-pathology/cytology methods and molecular biological TB-TMA methods detailed in the next sections.

The outline of the strategy for our approach is shown in Fig. 1. Sputum, samples were subjected first to smear techniques followed by Ziehl-Neelsen (ZN) staining. Tissue biopsies and lymph node FNAC went through histopathological/cytological techniques prior to ZN staining. ZN staining picks out all mycobacterial species. However, where the bacterial load in the sample is not enough, ZN staining may not be very effective; it is however the fastest method to screen for mycobacteria. Therefore, the samples simultaneously underwent bacterial isolation/ cultivation. Bacterial isolation/cultivation on Lowenstein-Jensen (LJ) medium is specific for mycobacteria and mycobacterial species can be determined through biochemical tests. The advantage here is that if the bacterial load of a sample is low, the bacteria are enriched for detection. Thus, any positive samples missed out by ZN staining can be captured by this method. This method however is slow, and takes about a week to obtain results. The positive samples can then finally be screened using the TB-TMA method which can type the organism down to the species level. The molecular biological methods are expensive and not without their own disadvantages. So, using this method at the final stage not only reduces the expenses, but also confirms the positives obtained through other methods and helps reject any false positives that might have been obtained. As we wanted to simultaneously compare the performance of the different methods, we also carried out the TB-TMA test on all 244 samples.

2.3. Processing of specimens for Ziehl-Neelsen (ZN) staining and culture

The sputum samples were directly smeared on slide and stained using ZN staining method. Tissue biopsies were first decontaminated using N-Acetyl-L-Cysteine, 2% sodium hydroxide and sodium citrate, PBS (pH 6.8). After samples were decontaminated, the smears were stained by the ZN staining method and were examined by trained technical staff for the presence of acid fast bacilli. The cultures were grown on the Lowenstein-Jensen (LJ) egg based medium as per the standard methods [2] for final identification of M. tuberculosis. The cultures were incubated for up to 8 weeks and were examined for growth of M. tuberculosis. An un-inoculated tube of LJ medium was used as negative control and *M. tuberculosis* A.T.C.C. H37Rv was used as positive control. Thereafter, molecular biology confirmation tests for *M. tuberculosis* were applied.

2.4. Histopathology and cytology

We chose the infected part of tissue. Around 5 mm size of tissue was transferred to cold 1xPBS, fixed using 4% PFA, and were classified according to the pathological state. The tissues were embedded in Tissue-Tek in a plastic mold and immersed in dry ice. All samples, wrapped in aluminum foil were placed in a labeled box to store at -80^{o}C.The blocks were sectioned using cryostat. Cryosection blocks were cut at thickness of 10 µm, placed on poly-lysine coated slides and dried at room temperature. The sections were fixed using acetone and staining was done by hematoxylin-eosine (H&E) method and viewed under light microscope (Olympus). At least 100 cells from the same tissue were measured. In each case, at least 10 fields per slide were selected and analyzed. As described above, the sections were stained using ZN staining method for viewing the bacteria.

FNAC of lymph node was performed using a 21-G needle. The tissue morphology was viewed under the microscope after haematoxylin-eosin staining. If caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells, or macrophages were seen, specimens were considered positive and were confirmed for mycobacteriosis (Goel et al. 2012). The fluid specimens were loaded on labeled slides using cytocentrifuge, chamber & blotter for each sample to be examined. Cell suspension (200ul) to a slide chamber and were spun at 800 rpm for 5 mins. The slides were carefully removed from cytocentrifuge and allowed to air dry prior to H&E and ZN staining.

2.5. *M. tuberculosis* confirmation via nucleic acid amplification (TB-TMA)

Testing was done using our Food and Drug Administration (FDA) approved TB-TMA test developed by Oncquest India Ltd., from the Central state of India. The TB-TMA method was used to detect M. tuberculosis. The method identifies infection with *M. tuberculosis* to species level and safeguards against false detection of bacteria other than tuberculosis or Non-tubercle mycobacterial (NTM) infections. This method, using the Gen-Probe direct test, was conducted according to the manufacturer's recommendation. In short, the decontaminated samples were subjected to isothermal enzymatic amplification of rRNA with DNA intermediates. Amplicon was detected with a fluorescent labelled DNA probe specific to rRNA of M. tuberculosis complex. As described earlier (Shamputa et al. 2013; Vlaspolder, et al. 1995) decontaminated lysates (50 mL) were used for target amplification. Every test was validated by comparing with a positive strain of M. tuberculosis culture (A.T.C.C. H37rv) as positive control and E. coli (A.T.C.C 35218), which has gram positive like morphology, as negative control.

III. Results

We used various methods to diagnose *M. tuberculosis* at our laboratory – AFB staining method which detects all mycobacterial species, cultivation of isolated bacteria on LJ medium, histopathology-cytology study, and TB-TMA molecular biology method. The results of each test determined how the sample was processed further (Figure 1).

Table 1 shows the different methods used and percentage positive samples detected by each method. Of the 244 samples tested, the highest percentage (81%) of samples was found to be Mycobacterium positive by histopathology study. These samples showed necrosis with caseating appearance along with bacteria that could be visualized by ZN staining and were considered to test further for mycobacterial infection. Microbiology methods of ZN staining and bacterial cultivation could both diagnose close to 50% samples to be positive for Mycobacteria. Final test using the TB-TMA method showed around 34% samples to be positive specifically for *M. tuberculosis*.

Next, suspect patients were divided into age-groups separated by 5 years from age 1 to 55. We placed patient suspects >55 years of age into one single group. Percentage of suspects diagnosed as positive in each age-group was calculated (Table 2). We had very few samples in the first three age groups, and none of these were found to be positive for *M. tuberculosis*. The largest number of suspects were in age-group >55 (64) followed by age group 20-24(32). However, it was in age-group 20-24 that highest percentage of positive diagnosis was made – around 56% of the suspect patients in this group tested positive for *M. tuberculosis*.

The suspect patients (with disclosed age) were further segregated according to gender. The total percentage of male and female suspect patients of all age who tested positive for *M. tuberculosis* was very similar (34.9 and 34.1% respectively) – Table 2. However, when the patients were segregated according to both age and gender, another picture appeared (Fig. 2, Table 2). The highest percentage of samples from male suspect patients that tested positive for *M. tuberculosis* were in the age-group 20-24 followed by age-group 25-29 (68.8 and 60% respectively). On the other hand, the highest percentage of samples from female suspect patients that tested positive for *M. tuberculosis* belonged to age-group 35-39 (60%) followed by age-groups >55 (45.5%) and 20-24(43.8%).

We further checked for gender differences for the different types of samples tested for *M. tuberculosis* (Table 3). Overall, the pulmonary samples had a higher percentage of positive diagnosis compared to the extra-pulmonary samples. When segregated by gender, the sputum samples tested were found to be similarly positive for both the genders. On the other hand, 67% of female BAL samples tested positive for M. tuberculosis, while only 9% male BAL samples were found to be positive.

Considering the extra-pulmonary samples – almost 50% of "Tissue" samples tested were found positive for *M. tuberculosis* in both the genders. Male and female urine samples were similar in percentage positive diagnosis. Higher percentage of male CSF and Pus samples were found to be positive for M. tuberculosis compared to female samples. On the other hand, higher percentage of female FNAC and "other tissues" samples tested positive for *M. tuberculosis*. Semen, menstrual blood, endometrium are all gender specific samples, and data can therefore only be reported for the specific gender. Interestingly, although menstrual blood and semen samples were found positive despite a small number of samples in each of these types, almost all endometrial samples were found negative for TB.

IV. Discussion

Pulmonary as well as extra-pulmonary tuberculosis is increasing globally. Extra-pulmonary involvement can be seen in more than 50% patients with HIV-tuberculosis co-infection. The risk of extra-pulmonary tuberculosis increases with advanced usage of immune-suppressants – as in case of cancer patients, and where body's own immune system is suppressed as in the case of HIV infection. Extra-pulmonary tuberculosis is associated with visceral lymphadenopathy, tissue abscesses, and negative tuberculin skin test [19–21].

TB in humans is caused by M. tuberculosis. Clinical symptoms similar to TB are produced by NTM species. It is therefore important that *Mycobacterium tuberculosis* be distinguished from NTM to direct the patients towards a quick, successful treatment.

In developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods like demonstration of AFB in smears has great importance but is prone to false negative results. Despite the development of various other methods, a single diagnostic tool for TB still remains insufficient, as each method has its own methodological advantages and disadvantages in the final diagnosis of infection to aid start of the therapy.

Through this work at Central lab, Indore, we show that a combination of diagnostic tools, including staining, histopathology and the TB-TMA molecular biology method, along with other classical microbiological and biochemical methods for diagnosis of infection with *M. tuberculosis* at species level, is the best method of achieving sufficient sensitivity and specificity for the diagnosis of *M. tuberculosis* in different type of samples.

All the samples from clinical suspects for TB went through screening for M. tuberculosis using the various methods. Different methods gave different percentage of positive results (Table 1). AFB smear method with ZN staining is limited by rather poor sensitivity and a need for 5000-10000 mycobacteria per mL of sample for the results to be positive[22] – our results were no different with about 51.2% samples found positive. This method is also limited by its recognition of both M. tuberculosis and NTMs. However, this method is definitely the first, most economical and the most rapid test towards TB diagnosis. The positive samples can immediately proceed to NAA tests for the confirmation of TB.

M. tuberculosis isolation and cultivation on LJ medium, although can detect samples with bacterial load of 10-100 bacilli per mL sample[22], is also known to be rather insensitive – as also is seen in our results, only 53% of samples were found positive in contrast to histopathology-cytology (81%) – and is also limited by the long time it takes to cultivate M. tuberculosis. However, when positive, isolation and cultivation of M. tuberculosis is considered "gold standard" for detection of TB, and thus this method retains its value despite the development of advanced NAA methods. This method also is valuable precursor to drug sensitivity testing (DST).

Histopathology-cytology is the often the primary method of choice, especially for samples from clinical suspects of extra-pulmonary TB [23]. In conditions like these, where there is lower load of M. tuberculosis, it can be difficult to detect them using AFB staining, and considering that culture methods take long time, histopathological methods can be faster in providing a first diagnosis [24,25]. In addition, we also

performed histopathology-cytology studies for samples from suspect cases of pulmonary tuberculosis. These were followed up by ZN staining of the AFB, bacterial isolation and cultivation and the TB-TMA test. More positive samples could be found using this method than ZN staining or cultivation alone (81%). Finally, using the TB-TMA method, we were able to identify the organism causing infection up-to the species level of M. tuberculosis and proceeded with drug sensitivity tests against the specific organism diagnosed as the infecting organism.

The prevalence of tuberculosis in the Central state of India has been reported earlier [4,17], however one of these studies explores pulmonary TB and the other one uses only bacteriological analysis. We decided to use our data for analyzing the prevalence of TB in the Central state of India and to see if any trends could be observed. It has been established that there is a relationship between the mortality, morbidity and diversity of TB with age-group of the patient[26]. Large prevalence surveys have showed that there is a gender bias in pulmonary TB, with more males being affected than females, and have suggested biological mechanisms behind this bias[27]. We therefore decided to check our data for any such age or gender bias.

Our data (table 2) shows that the patients most suspected to have TB were from age-group >55. This is not surprising considering that the immune status reduces with age. Two age groups – 20-24 and 35-39 had higher than average percentage of positive diagnosis for TB -the average percentage being around 30% (Figure 2a). Both the groups fall within the reproductive age-group (15-49) and thus it is not surprising to see these peaks. However, when the samples were also segregated based on gender (Fig 2b), the age-group 20-24 was found to have nearly 25% more males positive for TB as compared to females. The age-group 25-30 also had nearly 35% more positive males than females. On the other hand, age-group 35-39, with second highest percentage of positive diagnosis for TB, had 35% more females as compared to males. Male positivity for TB was also higher between the ages of 20 and 30. This analysis holds true after taking into account male and female specific samples (semen, endometrium and menstrual blood). It was surprising to observe this gender bias in the different age-groups. Whether this difference is due to biological or social reasons, remains to be seen.

The male positive diagnosis for TB fell dramatically after 30 years of age with a rise again after 55 years; for females it was more or less constant on either side of the peak at 35-39, with a slight rise after 55 years. The rise after 55 years of age could be related to fall in immunity due to aging.

Under the age of 15, we got no positives. The number of samples that we could obtain in this age group was low in the first place. Secondly, pediatric samples are rather difficult to diagnose especially due to difficulty in obtaining samples and due to lower load of tubercle bacilli.

With respect to different samples tested for TB, more pulmonary samples were found positive for TB as compared to the extra-pulmonary samples. It is known that females have significantly higher risk than men to have extra-pulmonary tuberculosis than men[28], so we further checked for gender differences for the different types of samples tested for *M. tuberculosis*. While more females were positive for certain type of samples (FNAC and 'other fluids'), more males were positive for others (CSF and pus). Considering the female specific samples, endometrium samples were largely negative for M. tuberculosis. Endometrial samples are often used to detect endometrial TB that can cause infertility in women[29], and we speculate on the possibility that these endometrial samples were collected from infertile patients and were sent for TB diagnosis to rule out endometrial TB. However, this conjecture needs to be confirmed in the future studies.

V. Conclusion

We demonstrate that, utilizing combinatorial methodologies is ideal for diagnosis of tuberculosis and the results of each methodology could be well correlated. Each of these methods has their own limitations along with possibility of their own false negative or positives. However, these methods when used together lend strong support for each other thereby supporting medical practitioners in the final diagnosis of mycobacterium infection and directing patients towards right internationally approved therapy against TB. We are thus able to help medical professionals make a more accurate decision on treatment at better speeds by utilizing the methods in combination for diagnosis. We are the first laboratory of central India that provides this type of personalized testing for M. tuberculosis to the patients clinically suspected of having TB infection. We also present a more accurate picture of TB between 2012 and 2014 diagnosed in our laboratory in the Central state of India. The age and gender bias needs to be studied more thoroughly.

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Method	Method characteristics	Positive (%)	Negative	Total
ZN staining	Positive for all Mycobacterial species	125 (51.2%)	119	244
Cultivation and isolation of M. tuberculosis	Positive for all Mycobacterial species; identification of species by biochemical tests	129 (53%)	115	244
Histopathology and Cytology	Tissue infection identified with microscopy associated with clinical tests	197(81%)	47	244
TB-TMA	Positive specifically for M. tuberculosis	84 (34.4%)	160	244

Table 1. Comparison of different approaches –AFB staining, bacterial cultivation, histopathology study and molecular (TB-TMA) method – for samples from the clinical suspects in Central state India (M.P.) tested for Tuberculosis.

Age groups of patients	Samples analyzed			Positive samples			Percent positive samples		
	Total	M	F	Total	M	F	Total	M	F
1-4	2	2	0	0	0	0	0	0	0
5-9	4	1	3	0	0	0	0	0	0
10-14	4	1	3	0	0	0	0	0	0
15-19	18	8	10	6	3	3	33.3	37.5	30
20-24	31	16	15	18	11	7	60	68.8	48.4
25-30	32	10	22	11	6	5	34.4	60	22.7
31-34	23	5	18	7	1	6	30.4	20	33.3
35-39	18	8	10	8	2	6	44.4	25	60
40-44	18	8	10	3	1	2	16.7	12.5	20
45-49	18	7	11	5	1	4	27.8	14.3	36.4
50-54	14	5	9	4	2	2	28.6	40	22.2
>55	54	32	22	19	9	10	35.2	28.1	45.5
Undisclosed	8	3	5	3	1	2			
Total	**244**	**106**	**138**	**84**	**37**	**47**	**34.4**	**34.9**	**34.1**

Table 2: TB diagnosis data segregated by age groups and gender.

		Pulmonary Samples		Extra-pulmonary Samples										
		Sputum	BAL	Tissue	Endo-metrium	Pleural Fluid	Other Fluids [a]	Semen	Menstrual Blood	CSF [a]	Pus [a]	Urine	FNAC [b]	Total
	Total	7	23	28	22	33	28	1	2	64	23	7	6	244
	F	4	12	17	22	13	17	NA	2	31	15	4	2	138
	M	3	11	11	NA	20	11	1	NA	33	8	3	4	106
MT+ve	**F**	3	8	8	1	5	6	NA	1	10	3	1	2	48
	M	2	1	5	NA	5	1	1	NA	16	4	1	0	36
MT+ve (%)	**F**	75	66.7	47	4.5	38.5	35.3	NA	50	32.3	20	25	100	--
	M	66.6	9	45.5	NA	25	9.1	100	NA	48.5	50	33.3	0	--

Table 3: Different samples tested for clinically suspected tuberculosis from the central state of India (M.P.) using the TB-TMA method.

N-1 two proportion test for small samples or Fisher's exact test was used as appropriate to calculate significance of association between TB positive samples and gender. MT: M. tuberculosis, M: male, F: female, NA: not applicable, MT+ve: Positive for M. tuberculosis. a: N-1 two proportion test for small samples ; b: Fisher's exact test. P-values: Other fluids – 0.06; CSF- 0.09; Pus – 0.07, CI ; FNAC- 0.066.

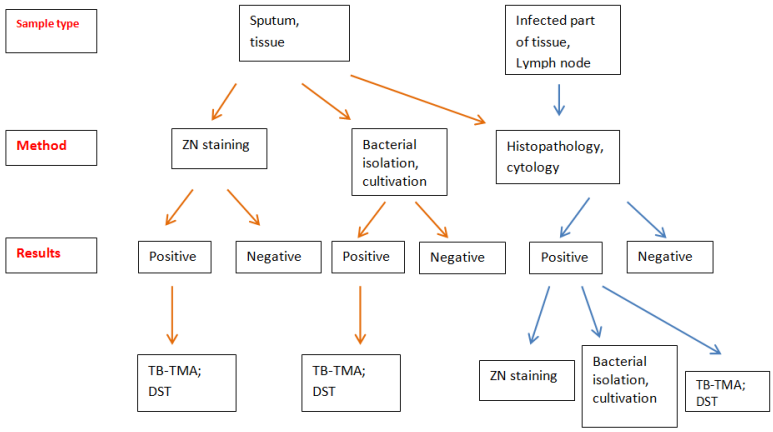

Fig.1: The strategy of using multiple methods to diagnose suspect samples for TB infection in conjunction with each other

a) b)

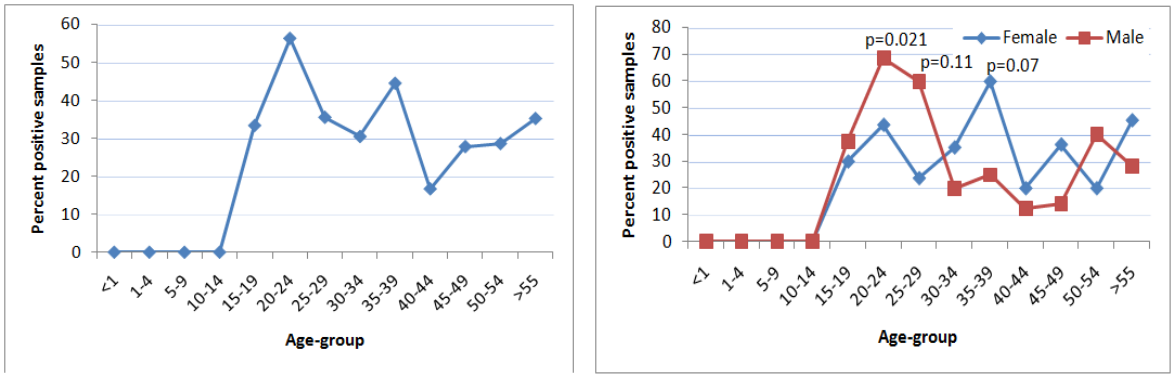

Fig. 2: a) Plot of age-groups against proportion diagnosed positive for M. tuberculosis. b: Plot showing proportion of samples from male and female patient suspects belonging to different age groups.N-1 two proportion test was used to check for significant differences between genders in each age group, and the p values are reported for samples with significant proportion difference between genders.