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Comparative Efficacy of Different Laboratory Techniques Used in Diagnosis of Tuberculosis in Human Population

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In order to test the efficacy of different laboratory techniques used in diagnosis of tuberculosis, 50 Tb cases along with 50 control cases in Faisalabad area were studied, using different laboratory tests. Mantoux's test showed no correlation with hematological parameters. Acid fast test (AFB-Test) was routinely used in labs, but there was still chances of contamination of sputum sample with a typical mycobacteria, so increasing the chances of error in the results. Chest radiograph was also a specific technique to some extent but in case of small children and other lungs diseases, it became unreliable and confusing test. When a comparative evaluation was made between different diagnostic tests, polymerase chain reaction (PCR) proved to be a very sensitive, highly specific, qualitative and quantitative technique for the confirmation of pulmonary tuberculosis. However, there was no correlation of PCR results with other hematological findings. Hematological studies showed significantly high level of erythrocyte sedimentation rate (ESR) in Tb cases (64.3 ± 38.2) ($P<0.00$) than in control cases (16.57 ± 5.26) were significantly increased (72.5 ± 39.9). Hemoglobin level (Hb) of Tb patients was significantly decreased (8.84 ± 2.33) than in control cases (11.8 ± 1.65). Total Leucocyte count (TLC) in Tb patients was significantly ($P<0.000$) higher (8882 ± 1650) as compared to control cases (62134 ± 1161.68). Differential leucocyte count (DLC) analysis of Tb cases in comparison with control cases showed significantly high ($P<0.000$), neutrophil count in Tb cases (69.4 ± 4.69) as compared to control cases (60.04 ± 5.15). Lymphocyte count in Tb cases was significantly decreased ($P<0.000$) (24.55 ± 3.64) than in control cases (32.39 ± 4.63) and eosinophil count in Tb cases was (2.16 ± 1.34) and control cases (3.2 ± 1.55) at probability level of (0.0004). Monocytes and basophil count was non-significantly different ($P<0.1127$; $P<1.00$) in Tb cases (3.7 ± 1.72, 0.08 ± 0.27) from control cases (4.38 ± 2.55, 0.08 ± 0.27). It may be concluded that PCR test is reliable, which may be employed in routine testing of Tb cases to safeguard against misdiagnosis.

Key words: Tb, *Bacilli tuberculi*, epidemiology, Mantoux/Tuberculine test, X-Ray analysis, microscopic smears, acid fast test, PCR technique, hemoglobin, hematology

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Introduction

Diagnosis of tuberculosis requires knowledge of the strengths and shortcomings of various diagnostic methods and experience in their use. No diagnostic method, by itself, can be relied on to confirm or rule out tuberculosis. Well tested diagnostic methods of chest radiograph, tuberculosis skin testing, smear and culture have recently been supplemented by rapid diagnostic test based on amplification of bacterial DNA. Failure of compliance can be a significant problem in patients who are homeless or drug abusers, or for various reasons cannot complete a course of therapy. Directly observed therapy is strongly recommended for these patients (Mc Dermott *et al*., 1997). About one third of the world population is infected with T*uberculi bacilli,* causing eight million new cases of tuberculosis and three millions deaths each year. A more complete epidemiology of Tb, will lead to a better identification of index cases and to a more efficient treatment of the disease. Recently, new molecular tools have become available for the identification of Mycobacterium Tuberculosis (MTb) strains, allowing a better recognition of transmission routes of defined strains. Both a standardized restriction fragment length polymorphism (RFLP) based methodology for epidemiological studies on a large scale and DNA amplification based methods that allows rapid detection of outbreaks with Mycobacterium Drug Resistant Strains (MDR), often characterized by high mortality rates, have been developed (Suffys *et al*., 1997).

Tb of man is caused by a group of very closely related species, forming the *Mycobacterium tuberculosis* complex. These include *M. tuberculosis* (cause of most infections) *M. bovis,* (endemic in cattle, spread to man by milk) and a typical mycobacteria (rare compared with human type), cause cervical lymph node infection in children, MTb is mostly transmitted in cough sprays in patients having overt pulmonary Tb and gain access to the body by inhalation of injective droplets. Initial lesion is usually in the lung from which organism reaches other organs through lymphatic and blood stream (Collier *et al*., 1998). The initial primay Tb occurs in the lungs but occasionally in the tonsil or alimentary tract. The primary infection differs from the subsequent infection in that the primary focus in lung, tonsil or bowel is almost invariably accompanied by caseous lesions in the regional lymph nodes. Post primary pulmonary Tb is the term used to describe lung disease, characteristic feature of which is tuberculous cavity which is formed when the caseated and liquified center of a tuberculous pulmonary lesion is discharged into bronchi. Extension of infection to pleura causes Tb pleurisy, when accompanied by effusion, develop tuberculous emphysema (Edwards and Bouchier, 1991).

DNA fingerprinting studies have established that exogenous re-infection occurs more often than previously believed. Drug resistant tuberculosis is an other form of Tb, which is usually defined as a resistant strain of MTb to isoniazid and rifampicin with or without additional drug resistance. Mycobacterium drug Resistant Tuberculosis (MDR TB) is an increase in many parts of the world. Secondary resistance is the result of preferential replication of mutants in patients receiving inadequate therapy. Primary resistance occurs when an untreated person is infected by a strain that is already resistant (Collier *et al*., 1998).

Tuberculosis may be partially explained by the higher rate of infection in the immunocompromised states associated with old age, renal failure, cirrhosis, malnutrition hematologic malignancies and AIDS. Different forms of extra pulmonary Tb are endobronchial tuberculosis, lymphatic, genital, kidney, arthritis, abdominal, intestinal, peritonitis, meningitis, disseminated and miliary tuberculosis (Wyngaarden and Smith, 1988). Common clinical features of all the types of tuberculosis includes anorexia, weight loss, sleep sweats, evening pyrexia, cough, sputum and hemoptysis. Case findings depends upon the common symptoms of disease, chest radiograph and sputum smear examination. These are the important and common methods of case finding in developing countries.

This study was undertaken to identify the status of tuberculosis in population of Faisalabad city and using different diagnostic methods to evaluate their relative efficacy.

Materials and Methods

This project was designed to determine the true situation of *Mycobacterium tuberculosis* infection in Faisalabad area, seek out the communities at a great risk and comparative evaluation of polymerase chain reaction (PCR) test with the existing remaining conventional diagnostic techniques.

Epidemiological data: In order to know the disease status of Tb in Faisalabad, retrospective descriptive epidemiological data were obtained from District Tb Hospital, Circular Road, Faisalabad. Previous data available at the hospital for the last decade (1989-98) were collected on a questionnaire having the following information:

(a) Date of admission of the case, (b) Name/sex of patient,(C) Age of patient, (d) Residential address, (e) Clinico-analytical epidemiological data.

The detailed information of 100 Tb cases admitted at the hospital was obtained on a predesigned questionnaire having following information.

Youngsters (under 23 years and unmarried) + ve/-ve or relapse case: (a) Name / Sex, (b) Age, (C) Father's Profession, (d) Source of income of family, (e) Person's status in the family, (f) Education, (g) Date of diagnosis, (h) Domestic animals, (I) Residential environment, (j) Family background related to Tb, (k) Dietary habits.

Male (married) + ve/-ve or relapse case: (a) Name, (b) Age, (C) Address, (d) Profession, (e) Total income, (f) Working hours, (g) Date of marriage, (h) Number of children, (I) Dietary habits, (j) Date of diagnosis, (k) Type of domestic animal if any, (l) Residential environment, (m) Family background related to Tb.

Female (married) + ve/-ve or relapse Case: (a) Name, (b) Age, (C) Address, (d) Type of official work/home assignments, (e) Husband's profession, (f) Total Income, (g) Date of marriage, (h) Number of children, (I) Dietary habits, (j) Date of diagnosis, (k) Type of domestic animal if any, (l) Residential environment, (m) Family background related to Tb.

Diagnostic studies

Selection of patients: Fifty (50) clinical Tb cases from District Tb Hospital, Circular Road, Faisalabad were selected for diagnostic tests along with 50 control cases.

Mantoux/tuberculin test: The tuberculin skin test was used based on principle given by Collier *et al*. (1998).

In the Mantoux/tuberculin test, an exact amount (usually 0.1 ml) of tuberculin (2Tu 0.1 ml^{-1}) was injected intracutaneously in the forearm, after 72 hours, infection site was palpated and measured induration as transverse diameter. Results were noted as an induration of 10mm diameter measured transversely indicated a positive reaction (Collier *et al*., 1998)

X-Ray analysis: In disease, around the area of infection, activated macrophages aggregate so, that a compact palisade, which is many cell thick, is formed to produce the characteristic lesion of tuberculosis and many other chronic infections, namely the granuloma. The apparatus used included, X-ray machine, Illuminating screen and the method employed included observation of anteroposterior chest radiographs on illuminator. Results were recorded according to the presence or absence of radiopaque characteristic lesions of Tb on Apex, center and base regions of both of the lungs in case of pulmonary tuberculosis patients versus control cases (Collier *et al*., 1998).

Microscopic smear examination: Acid fast bacilli (AFB) Staining/Ziele-Neelsen Staining. The staining technique is based on

the resistance of Mycobacteria to decolourization by acid or a mixture of acid and alcohol after staining by arylmethane dyes i.e., acid fastness' (Colliers *et al.*, 1998).
Mycobacterium were stained bright red. Results were noted on the basis of presence or absence of acid fact bacilli. The sputum smears containing acid fast bacilli were called + ve slides while others as –ve (Merchant and Packer, 1984).

Polymerase chain reaction (PCR) technique: PCR is based on the principle of amplification of specific DNA segment *in vitro*, after separating DNA stands by heating, then annealing to an excess of short synthetic DNA primars that flank the region to be amplified in the presence of taq-polymerase and dNTPs (Lehninger *et al.*, 1993). Digestion and decontamination of specimens were done by (Maniatis *et al.*, 1982).

PCR amplification: PCR amplification was done using apparatus such as Multi tube vortexer (Mickle apparatus); PCR Machine (thermocycler) PTC-100 programmable thermal controller, Microdispensors with tips, PCR tubes and the reagents (50uL total cocktail> used were: 10μl of PCR product, 5 μl reaction buffer containing, 10mM Tris-HCl (pH 8.3), 50mM KCl (potassium chloride), 1.5mM $MgCl_2$ (magnesium chloride), 0.01% gelatin, 0.2mM (0.2μL) dNTPs (deoxynucleotide triphosphates) dATP, dGTP, dTTP, dCTP, dNTPs – ABI Master piece, 500mg dissolved in autoclaved distilled H_2O, 0.2 μl-primers (each) available in commercial packing of company GENOSYS; 2μ (lμl) of Taq Polymerase. Taq polymerase was extracted from a thermostable bacteria called Thermus equatica and donated by the Restriction lab of CEMB, Lahore, 10 μl Genomic DNA of clinical sample of MTb Template, 10μl of Genomic DNA of Mycobacterium species other than MTb (NTM or MOTT), Distilled H_2O, to make final volume 50μl.
Mycobacterium tuberculosis insertion sequences IS 986 were used for amplification which was of 541 bP. The 33.6μl of distilled H_2O was added to all PCR tubes, then added 5.0μl of reaction buffer, containing 10mM Tris-HCl (pH, 8.3). 50mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin was added to all PCR tubes individually; 0.2mM (0.2μl) of dNTPs (dATP, dGTP, dETP, dGTP) was poured in each above PCR tube using another tip (0.2μl contained 20 p mole); 2μl of Taq polymerase was dispensed off in all PCR tubes, individually; 0.2μl of each primer named as Pt-8 and Pt-9 correspond to bP 105-124 (S-GTGCGGATGGTCGCAGAGAGAT-3) and Pb 626-645 (5-CTCGATGCCCTCACGGTT CA-3) respectively of TS986 sequence were dispensed in all PCR tubes, using different tips again; 10 μl of isolated DNA from each sample was added in all tubes at last for every sample tip of microdispensor was changed to avoid contamination. In –ve control PCR tubes, one was devoid of DNA containing all other PCR cocktail and other contained 10μl of genomic DNA of NTM (non-tuberculus microbacterium) + ve control PCR tubed contained 10μl of DNA of clinical sample of MTb (*Mycobacterium tuberculosis*); After vortexing the samples were shifted to thermocycler (PCR machine) and programmed as 2 minute initial denaturation at 95 °C 1 minute annealing at 55 °C and 30 seconds of primer extension at 72 °C. After 40 cycles final extension was done for 2 min at 72 °C (Idrees *et al.*, 1998).

Detection of PCR products: The gel containing 541 bp fragments, was visulaized by (UV transillumination after making comparison with markers (Maniatis *et al.*, 1982).

Biochemical studies: Complete blood picture (CBP) of 50 (Ffity) clinical Tb patients and 50 control cases was done by using following tests.

Erythrocyte sedimentation rate (ESR) test: Westergren method was used to perform ESR test. ESR, also known as sed rat, is a non-specific lab. Test does not give the physician information about a specific disease but they indicate presence of acute or chronic inflammation. Level of red blood cells was read as ESR (Kolmer *et al.*, 1951; Baker and Silverton, 1982).

Hemoglobin level (Hb level) detection: Hemoglobin (Hb) in the sample is oxidized in the presence of ferricyanide to hemoglobin (Hi, also called methemoglobin), which then reacts with cyanide at pH 7.2, producing hemoglobin cyanide (Hi CN or cyanmethemoglobin). Reaction of all haemochromogens is completely developed in 3 minutes, at room temperature. Readings are performed at 620 nm.
Homogenized the samples thoroughly before use. After setting the instrument at zero with reagent, 20 μl of each homogenized blood sample in a clean dry micro-pipette was mixed with 5 ml of Hb reagent, previously kept in cuvette, rinsing three times with reagent, mixed by inversion and kept for 3-5 minutes in stand. Then read the absorbance at λ=620nm after keeping the cuvettes one by one in the slot of colorimeter. Absorbance was carefully noted and recorded. Hb level measured by multiplication of sample's absorbance with factor.

Hb level of unknown sample =
Absorbance of unknown sample x factor
(Wedding and Toenjes, 1992).

Total leucocyte count (TLC): For TLC counts, 3% acetic acid (CH_3COOH) is used which hemolyses red blood cells and preserves white cells. The dilution of the blood to diluting fluid is 1:20. (Wedding and Toenjes, 1992)

Differential leucocyte count (DLC): For this purpose polychromatic stains were used each having different staining character. Some cellular structures except one type of dye, while other cellular structures are stained with another. Basic part of cell (cytoplasm) is stained by acidic portion of stain, giving pink appearance and acid part of cell (nucleic acid) is stained by basic portion of stain giving it purple to blue appearance, according to the type of stain used. On the basis of shapes of nuclei, size of cells and granulation, assign them different names like neutrophil, eosinophil, basophil monocyte or lymphocytes. The differential count gives only the relative number (percentage) of the different type of white blood cells (Wedding and Toenjes, 1992; Kolmer *et al.*, 1951; Baker and Silverton, 1982).
The experimental data relating to all laboratory methods used to diagnose tuberculosis in human beings was subjected to statistical analysis (Steel and Torrie, 1980)

Results and Discussion

Epidemiological studies spread over ten years were conducted in order to know the ways of transmission of disease in the community and impact of control measures. Seasonal trends of reported Tb cases were also noted, which showed a consistent remarkable increase from the month March to September, with highest trends in July and August. From 1989-96 there was endemic situation of Tb but remarkably increased in 1997 and 1998 (Table I).
Relative incidence (RI) of Tb in males and females, reported in each year was also calculated, showing high mean relative incidence of Tb in males (63.14%) as compared to females (36.86%) (Table I). These findings were substantiated by the findings of Collier *et al.* (1998), who reported that the Tb rate was significantly higher in males than in females as the males were more exposed to the outer environment, like male subjects working mainly as miners, were exposed to silica dust.
Age based epidemiology increased Tb incidence in age group from (15-39 years), with a mean age for Tb was 33 years. An average age for tuberculosis was 35 years, as reported by Follador *et al.* (1991). The highest incidence of disease was reported in males above 30 years. Caminero *et al.* (1995) carried out the distribution of tuberculosis, the Tb incidence peaked in age group 40-49 and 30-39 years.

Mantoux's test: Of the total observed clinical Tb cases, 54% cases showed a positive Mantoux's test. Thirtyfour percent (34%) were male Tb cases and 18% were female cases, so females showed less incidence of positive test. These findings were exactly in accordance with the findings of Collier *et al.* (1998), who stated that the positive Mantoux's test was shown mostly by males than the females patient. He defined the positive Mantoux's test > or = 10mm in duration. The major reasons for not showing the positive Mantoux's test might be the immunosuppression due to the previous or current antimicrobial treatment. A positive response to skin testing with tuberculin occurred only between 3 and 8 weeks after primary infection.

Furthermore, weak or negative reaction did not exclude a diagnosis of tuberculosis. Some persons were intrinsically poor reactors and in others reactivity was depressed due to advanced disease, old age malnutrition and immunosuppression, including HIV infection. Janis *et al.* (1996) declared a positive PPD (purified protein derivative) response of > or = 10mm induration. Independent risk factors for + ve, PPD test included age more than 55 years. Male sex with hypertension, HIV infection, current steroid use, history of cancer were associated with − ve PPD response. All Mantoux's test + ve cases (54%) showed + ve chest radiograph (54%); 6% AFB + ve and 12% PCR + ve results. Moreover the Mantoux − ve cases were all + ve (46%) by X-ray, 12% were AFB + ve and 20% PCR + ve showing no significant relationship between these tests. On the analysis of hematologic tests performed, the ESR values of Mantoux's + ve cases were slightly higher (65.551 ± 41.216) than −ve case (62.826 ± 34.77) but the increase was statistically non significant with a probability of 0.8069 (Table 2). Chan *et al.* (1990) elevated ESR and Mantoux test were valuable indicators for MTb infection.

Hb level of Mantoux's Test + ve cases was low (9.524 ± 2.766) than −ve cases (8.065 ± 1.44). The difference of Hb level between both +ve and −ve cases were statistically significant ($P<0.0213$) (Table 2). The total leucocyte count (TLC) +ve cases were 8144.81 ± 1917.74, neutrophil count (68.791 ± 5.758), lymphocyte count (24.77 ± 3.516), eosinophil count (2.111 ± 1.187), monocyte count (3.444 ± 1.423) and basophil (0.109 ± 0.315). While of −ve cases, TLC were 8660.86 ± 1252.26, neutrophil count (69.21 ± 5.38) lymphocyte count (24.43 ± 3.80), eosinophil count (2.30 ± 1.49), monocyte count (3.96 ± 1.87) and basophil count (0.043 ± 0.21) (Table 2). The probability values were 0.2594 for TLC, 0.95 for neutrophil, 0.7421 for lymphocyte, 0.608 for eosinophil, 0.2776 for monocyte and 0.3744 for basophil, showing a non-significant difference between these parameters.

Chest radiograph (X-Ray): All studied cases were showing abnormal radiological shadows on X-Ray with 18% AFB + ve, 34% PCR +ve and 54% Mantoux's test +ve. Many of these cases had different extent of cavities with different localities on lung area. Some had healed cavities with fibrosis and calcification. When the evaluation was made on the basis of hematological parameters, an increase of ESR values were seen in 100% cases (63.3 ± 38.2) in X-Ray +ve cases, while (16.57 ± 5.26) in −ve cases with a probability ($P < 0.0000$ showing a significant difference between both ESR values. Hemoglobin level (Hb level) in X-Ray + ve cases was decreased (8.84 ± 2.33) than X-Ray -ve cases (11.81 ± 1.65) while probability calculated was 0.0000 showing highly significant difference. According to the study of Malhotra *et al.* (1996), Pulmonary Tb cases were 80.6% + ve on Chest Skiagrom with 27.5% + ve by Mantoux test and 30.61% sputum +ve. ESR of all patients was raised. According to another report, mean age for Tb was 33 years, raised ESR, mild anaemia and histological examination gave + ve diagnosis in 100% cases with 56% AFB + ve cases (Wells *et al.*, 1986).

Data analysis (Table 2) revealed a remarkable significant increase of total leucocyte count in + ve cases (8882 ± 1650) while in −ve cases (6212.4 ± 1161.68) with probability 0.000. A significant result with probability 0.000 was obtained when neutrophil count was compared between X-ray + ve (69.4 ± 4.69) and −ve case (60.04 ± 5.15).

Lymphocyte count in -ve cases was slightly decreased (24.55 ± 3.64) than in −ve cases (32.39 ± 4.63), $P < 0.00$ and result was significant statistically. Eosinophil count in + ve cases was (2.16 ± 1.34), while in −ve cases (3.2 ± 1.55), so significant difference was found. Monocyte count also showed no significant difference in +ve (3.7 ± 1.77) and −ve cases (4.38 ± 2.55) while probability was 0.1127. Basophil count of +ve and −ve cases was same (0.08 ± 0.27) with probability 1.00, showing no statistical difference of both values. These findings were further corroborated as drawn by Jain *et al.*, (1991) who observed clinical and radiological differences and compared + ve and −ve case of pulmonary Tb. He noticed anaemia, leucocytosis, raised ESR and abnormal radiological shadows in +ve pulmonary Tb cases. Onwubalil and Scott (1988) declared no correlation between cellular immunity, radiological extent of disease, TLC and ESR.

Acid fast bacilli staining test (AFB test): 18% of all clinical Tb cases were found + ve with high incidence in males (12%) than females (6%); 6% of AFB +ve cases, were Mantoux's Test +ve also, while 50% were Mantoux's test + ve but AFB −ve showing no correlation of immunologic response to number of bacilli present. All cases were X-Ray + ve irrespective of AFB test + ve or −ve; 12% cases were PCR +ve which were also +ve by AFB test but 20% PCR test +ve were shown by the AFB −ve cases. 12% cases among 18% were +ve by both PCR and AFB test, 6% AFB +ve were PCR test −ve. So AFB test +ve but PCR test −ve showed, high chances of lab-contamination with other mycobacteria. The −ve AFB and +ve PCR test showed a low number of mycobacteria ($< 10^4$/ml) which was not detectable by simple AFB staining technique (Collier *et al.*, 1998). When a comparison of hematological tests was made between AFB +ve and −ve case, the ESR values of +ve cases were decreased (51.22 ± 36.85) then −ve cases (67.14 ± 38.38) with probability level of 0.2622 showing non-significant difference. Hb level of AFB-test +ve cases was slightly increased (9.16 ± 2.506) than −ve cases (8.751 ± 2.359), showing non-significant difference ($P<0.5984$). Total leucocyte count (TLC) was (8211.11 ± 1076.6) in +ve cases while (8419.75 ± 1756.67) in −ve cases, with non significant difference ($P<0.7351$) (Table 2).

Neutrophil count in + ve case was (69.66 ± 5.3) while in −ve case (69.34 ± 4.6) so non-significant difference was shown ($P<0.8531$). Lymphocyte count in +ve cases was (23.88 ± 4.16) and in −ve cases (24.7 ± 3.5) showing non-significant difference ($P<0.5427$). Eosinophil count in AFB +ve cases was (2.22 ± 0.971) and in −ve cases (2.82 ± 3.24) so, non significant difference was present ($P<0.5527$). Monocyte count in AFB +ve was (4.11 ± 2.26) and in −ve cases (3.7 ± 1.63) so ($P<0.4315$) non-significant difference existed. Basophil count in +ve cases was (0.1111 ± 0.333) while in −ve cases (0.073 ± 0.263) with $p<0.711$, so non significant difference was present. In all Tb cases whether AFB test +ve or −ve no difference in hematologic parameters were shown. So a Tb case can not be declared AFB test +ve or −ve on the basis of results of hematological parameters (Table 2).

Polymerase chain reaction (PCR) test: 34% of all observed clinical Tb cases showed +ve PCR-test result, in which 24% were males and 10% females. So mostly males showed a high ratio of PCR test positively than females. All PCR test ± ve or −ve were X-ray ±ve showing no relationship with extent of cavities at lung areas (Table 2).

16% of PCR test were ±ve and Mantoux's test ± ve, while 40% of PCR test were −ve but Mantoux test ±ve also, no correlation of immunological responses to PCR test was detected. 12% cases were AFB + ve and PCR test ± ve, 6% cases were AFB +ve but PCR test −ve because, AFB test could not distinguish between typical (MTb) and Atypical (MOTT or NTM) Mycobacteria (Collier *et al.*, 1998). 22% PCR test +ve cases (34 - 12 = 22%) were AFB

Table 1: Relative incidence of Tb in males and females reported at district Tb Hospital, Circular Road, Faisalabad (1989-98)

	Males		Females		
Years	No.of cases	Relative incidence %	No.of case	Relative incidence %	Total
1989	250	60.38	164	39.62	414
1990	280	64.96	151	35.04	431
1991	336	65.36	178	34.64	541
1992	291	67.36	141	32.64	432
1993	258	60.28	170	39.72	428
1994	289	60.33	190	39.67	479
1995	261	59.31	179	40.69	440
1996	282	62.11	172	37.89	454
1997	383	63.62	218	36.38	601
1998	399	66.05	205	33.94	604
Total	3029	63.14	1769	36.86	4797

Table 2: Statistical analysis of different laboratory methods and hematological parameters used to diagnose tuberculosis in human population

		Laboratory methods				Mean ± SD values in hematological parameters							
Subjects	No.of cases.	Mantoux test%	X-Ray %	AFB test%	PCR test%	ESR mm/ hr^{-1}	HB lavel g dl^{-1}	TLC cm m^{-1}	Neutrophil %	Lymphocytes %	Eosinophil %	Monocyte %	Basophils %
Control subjects													
Males	33	8	0	0	0	18.90	12.39	6382.42	60.84	32.07	3.06	3.39	0.06
(+ ve)						±5.02	±1.52	±1108.26	±5.124	±4.64	±1.56	±2.37	±0.422
Female	17	8	0	0	0	15.92	10.67	5952.94	57.88	33.29	3.35	5.24	0.12
(+ ve)						±4.47	±1.28	±1238.00	±5.34	±4.98	±1.49	±2.75	±0.33
n	50	-	-	-	-	-	-	6213.4	60.04	32.39	3.2	4.78	0.08
								±1161.68	±5.15	±4.63	±1.55	±2.55	±0.27
Tb cases													
Males	32	40	64	12	24	59.65	9.48	8470.31	70.27	24.07	1.97	3.44	0.031
(+ ve)						±37.06	±2.52	± 1648.21	±4.00	±3.62	±1.78	±1.54	±0.078
Female	50	18	36	6	10	72.50	7.74	8225.55	67.83	25.38	2.5	4.11	0.11
(+ ve)						±39.9	±1.50	±1689.82	±5.57	±3.51	±1.58	±1.87	±0.032
n	50	-	-	-	-	-	-	8882	69.4	24.55	2.16	3.7	0.08
								±1650	±4.69	±3.64	±1.34	±1.77	±0.27
PCR test													
PCR + ve	17	16	34	12	34	79.17	8.96	9055.88	71.23	23.41	1.76	3.47	0.117
Male					24	±34.03	±34.03	±1385.85	±3.78	±3.84	±1.2	±1.33	±0.332
Female					10								
PCR-ve	33	40	66	6	0	56.41	8.80	8029.09	68.45	25.21	2.36	3.28	0.060
						±37.92	±2.55	±1676.32	±11.8	±3.40	±1.39	±1.97	±0.242
AFB test													
AFP + ve	9	6	18	18	12	51.22	9.166	8211.11	69.616	23.88	2.22	4.11	0.111
Male				12		±36.85	±2.506	±1076.67	±5.315	±4.16	±0.971	±2.26	±0.333
Female				6									
AFB - ve	41	50	82	0	20	67.146	8.755	8419.75	69.34	24.7	2.82	3.7	0.073
n	50					±38.380	±2.395	±1759.67	±4.61	±3.50	±3.24	±1.631	±0. 2 6 3
						(P<0.2622)		(P<0.73531)	(P<0.3531)	P<0.5427	P<0.5527	P<0.4315	P<0.0711
Mantoux test													
Mantoux													
+ ve	27	54	54	6	12	65.551	9.524	8144.81	68.791	24.77	2.111	3.444	0.109
Male		34				±41.216	±2.766	+1917.74	±5.758	±3.516	±1.187	±1.423	±0.315
Female		18											
Mantoux													
- ve	23	0	46	12	20	62.826	8.065	8660.86	69.21	24.43	2.30	3.96	0.043
n	50					±34.77	±1.44	±1252.26	±5.38	±3.80	±1.49	±1.87	±1.21
						(P<0.8069)	(P<0.0213)	(P<0.2594)	(P<.65)	(P<.7421)	(P<0.608)	(P<0.608)	(P<0.3744)

Table 3: Statistical analysis of different biochemical/hematological parameters of control cases (Age based)

Age grouping (years)	ESR (mm hr^{-1})	Hb level (g dl^{-1})	TLC/ cmm	Neutrophil (%)	Lymphocytes (%)	Eosinophil (%)	Monocyte (%)	Basophils (%)
(12-22) n = 14	14.13	12.11	6090.71	57.5	34.14	3.07	4.78	0.14
S.D	±4.853	±1.86	±1348	±7.01	±5.9	±1.89	±2.69	±0.36
(23-32) n = 12	18.52	11.83	6658.33	60.58	32.33	2.66	4.5	0.083
S.D	±4.316	±1.68	±999.58	±4.55	±3.22	±1.15	±2.33	±0.29
(33-42) n = 7	17.16	12.9	6350.00	61.00	33.16	2.66	3.16	0.00
S.D	±4.49	±1.96	±1143.67	±6.6	±7.9	±2.16	±2.71	0.00
(43-52) n = 8	19.25	11.31	6462.50	59.87	31.62	3.63	3.75	0.00
S.D	±8.04	±1.43	±1506.82	±2.74	±2.87	±0.74	±3.11	0.00
(53-62) n = 5	16.0	11.8	5820.00	60.2	30.8	4.40	6.00	0.200
S.D	±3.39	±0.77	±739.594	±6.18	±1.64	±1.14	±2.44	±0.47
(63-72) n = 6	14.4	10.4	5550.0	62.8	29.6	3.4	3.8	0.00
S.D	±6.58	±0.41	±360.55	±1.92	±1.67	±1.3	±1.7	0.00

(P<0.05)

Table 4: Statistical analysis of different biochemical/hematological parameters of Tb cases (Age based)

Age grouping (year)	ESR (mm hr^{-1})	Hb level (g dl^{-1})	TLC/ (cmm)	Neutrophil (%)	Lymphocytes (%)	Eosinophil (%)	Monocyte (%)	Basophils (%)
(12-22) n = 11	67.63	9.014	9182.72	68.54	25.45	2.09	2.09	0.00
S.D	±39.59	±3.007	±1686.86	±4.16	±2.94	±1.22	±1.45	0.00
(23-32) n = 12	77.92	8.150	8008.33	69.83	24.08	2.08	3.67	0.25
S.D	±39.17	±1.59	±1778.38	±4.76	±4.29	±1.24	±1.49	0.45
(33-42) n = 7	47.14	9.74	8085.71	67.87	25.54	2.86	3.57	0.14
S.D	±32.50	±3.57	±2152.65	±5.82	±3.05	±2.19	±2.22	±0.38
(43-52) n = 8	61.37	9.45	9025.00	71.25	23.12	1.75	3.75	0.00
S.D	±40.43	±2.11	±989.94	±5.06	±4.42	±1.28	±1.75	0.00
(53-62) n = 5	98.00	9.4	7540.00	69.6	25.2	1.80	3.4	0.00
S.D	±18.90	±1.9	±1052.61	±3.36	±3.27	±1.1	±0.89	0.00
(63-72) n = 6	42.33	7.97	8066.6	69.16	24.16	2.33	3.66	0.00
S.D	±29.50	±1.06	±592.06	±5.98	±3.82	±1.03	±2.7	0.00

$P < 0.05$

test –ve because PCR being highly sensitive and specific could detect MTb in number 10^2/ml of given sample which could not be detected by simple AFB staining test (Collier *et al*., 1998). Fusegawa *et al*. (1996) evaluated the clinical diagnostic value of PCR assay for detection of MTb. PCR test lead to rapid diagnosis of Tb. PCR test was important to examine samples especially when these were smear –ve. Noordhock *et al*. (1995) declared the specificity and sensitivity of PCR 99.8 and 92.1%, respectively. Rapid and simplified PCR was slightly more sensitive than culture method (Zambardi *et al*., 1995).

By the comparative evalution of hematological parameters of PCR test +ve and –ve case, it got clear that there was a significant difference ($P < 0.0469$) of erythrocyte sedimentation rate values between PCR ±ve (79.17 ± 34.03) and –ve cases (56.41 ± 37.92). Hemoglobin level was not significantly ($P < 0.8239$) decreased in PCR –ve (8.80 ± 2.55) than PCR test +ve cases (8.96 + 1.99). However, there was a significant ($P = 0.0455$) difference of total leucocyte count (TLC) between PCR +ve cases (9055.88 ± 1385.85) and (8029.09 ± 1676.32) PCR –ve cases. Neutrophil count of PCR +ve case (71.23 ± 3.78) was non-significantly ($P < 0.0876$) increased than PCR –ve case (68.45 ± 11.8), lymphocyte count was also non-significantly decreased ($P < 0.096$) in PCR +ve (23.41 ± 3.84) than PCR –ve (25.21 ± 3.4). When eosinophil count was compared, there was a non-significant ($P < 0.175$) difference between PCR +ve (1.76 and 1.2) and PCR –ve cases (2.36 ± 1.39). Monocyte count in PCR + ve (3.47 ± 1.33) and PCR –ve (3.28 ± 1.97) was not significantly different ($P < 0.5063$). Basophil count difference was also statistically non-significant ($P < 0.2718$) between PCR +ve (0.117 ± 0.332) and PCR –ve cases (0.060 ± 0.242) (Table 2).

From these analysis it was clear that no assessment about PCR test could be made on the basis of hematological parameters and PCR was independent, sensitive and specific technique.

AFB-Test could detect 5,000-10,000 microbacteteria/ml of sample, while culture could detect 10-100 organism ml^{-1} but it was lengthy and time consuming method, DNA amplification by PCR and related methods were more sensitive and could detect 100 organisms/ml of clinical samples within short time (Collier *et al*., 1998).

Biochemical tests

Erythrocyte sedimentation rate (ESR) test: Erythrocyte sedimentation rate of control cases were 14.13 ± 4.853, while of Tb cases were 67.63 ± 39.59 the ESR value of Tb patients was significantly higher ($P < 0.0000$) than control cases (Table 3 and 4). Fishman (1980) reported that there was a high ESR level in patients having advanced active Tb, but irrelevant increase in ESR was common, limiting its usefulness. ESR had long been used as an indicator of activity in chronic pulmonary Tb. ESR test was a non-specific test, could not give the physician information about a specific disease and presence of acute or chronic inflammation. The speed at which erythrocytes settled down out of solution was dependent on many factors like size of erythrocyte, their concentration, shape and composition of plasma proteins in the sample. The proteins that caused erythrocyte to clump and increased the sed-rate included fibrinogen and globulins, which affects the surface hydration (Wedding and Toenjes, 1992). Onwubalil and Scott (1988) stated many abnormalities during tuberculosis, one of them was raised ESR with hyperglobulinemia. According to Hannisdal and Engan (1991) ESR was only a prognostic factor. In tuberculosis patients, ESR values were significantly raised (Jurik *et al*., 1982; Laudet *et al*., 1980; Sharp and Goldman, 1987; Syrjala, 1986). When data were analyzed on the basis of age, age group (12-22) of control cases showed ESR (14.13 ± 4.85) and Tb cases (67.63 ± 39.59), so there was a significant increase of ESR in Tb cases ($P < 0.0002$). Group II (23-32) of control subjects showed ESR (18.52 ± 4.3) while, Tb cases showed ESR (77.92 ± 39.17) ($P < 0.00$) so, difference was statistically high (Table 3 and 4).

Group III (33-42) of control subject had ESR (17.16 ± 4.49), while Tb cases had (47.14 ± 32.5), P (0.0345) so significant difference was present in group IV (43-52), had ESR (19.25 ± 8.04), while Tb cases had ESR (61.37 ± 40.43), $P < 0.0109$, showing significant difference.

Group V (53-62) of control subjects showed ESR (16.0 ± 3.39), while Tb cases showed ESR (98.0 ± 18.9), $P < 0.000$ highly significant difference was shown. Group VI (63-72) of control subjects showed ESR (14.4 ± 6.58) while Tb cases (42.33 ± 29.5), $P < 0.0563$, the difference of values was non-significant. So, most significant difference of ESR values was shown by age group (23-32) and (53-62) (Table 3 and 4). When the data was analyzed on the basis of sex, there was a statistically non-significant ($P < 0.113$) increase in ESR values of control males (18.9 ± 5.02) than in control females (15.92 ± 4.47). According to the standard values, the ESR values were relatively higher in normal females than in normal males (Kolmer *et al*., 1951).

In case of Tb patients, the ESR values of females were significantly ($P < 0.00$) increased (72.50 ± 39.9) than males (59.65 ± 37.06).

Hemoglobin (Hb) level: After analysis of data (Table 3 and 4) the Hb level of control cases was 12.11 ± 1.86, while of Tb casses was significantly decreased ($P < 0.000$) (9.014 ± 3.007). An iron deficiency in Tb patients was reported by (Luntz and Bogie, (1995), Wells *et al*. (1986), Baynes *et al*. (1986) and Jain *et al*. (1991). According to Collier *et al*. (1998), iron was essential for the growth of mycobacteria both *in vivo* and *in vitro*, Mycobacteria had evolved a sophisticated system for scavenging iron from the environment and their eukaryotic hosts. Iron was necessary for the bacterial growth and for antibacterial defense. A great part of intracellular iron was bound to special proteins, particularly Ferritin, some iron was complexed to sulphur and haem proteins. The supply of intracellular iron was, therefore, controlled by density of surface expressed transferrin receptor. Bacteria mobilize iron by releasing it from host iron binding proteins or by means of specialized iron binding proteins, the siderophores or by combination of both. Age based analysis showed age group 1(12-22 years) of control subject had Hb level (12.11 ± 1.86) while, Tb patients had Hb level (9.04 ± 3.007), ($P < 0.0047$) so, showing significant difference. Age group II (23-32 years) of control subjects showed Hb level (11.83 ± 1.68) while Tb patients showed (8.15 ± 1.59) expressing ($P < 0.00$) most significant difference. Age group III (33-42 years) of control subjects showed Hb level (12.9 ± 1.96), while Tb patient had Hb level (9.74 ± 3.57), ($P < 0.075$) so, statistically, difference was non-significant.

Age group IV (43-52years) of control subjects showed Hb level (11.31 ± 1.43), while Tb cases showed (9.47 ± 2.11) (P< 0.0619) so, non-significant difference was shown by this age group. Age group V (53-62) of control subjects showed Hb level (11.8 ± 0.77) while, Tb cases showed Hb level (9.4 ± 1.9), P<0.0353, so a significant difference was shown. Group VI (63-72) of control subjects showed Hb level (10.4 ± 0.41) while Tb cases showed Hb level (7.97 ± 1.06), so P< 0.001, showing significant difference between Hb values (Table 3 and 4). So statistically significant difference of Hb level was expressed by three age groups, like 23-32, 53-62 and 63-72 years. It means that among these above three age groups there was a significant drop of Hb level in Tb patients as compared to normal ones. When the data were analyzed on the basis of sex, the control males showed high Hb level (12.39 ± 1.52) as compared to females (10.67 ± 1.28) P<0.11 (Table 2), showing non-significant difference. Hb level was greater in case of males than females. Hb level in different age group was also different (Kolmer *et al.*, 1951). In case of Tb patients males had Hb level (9.48 ± 2.52) while females (7.74 ± 1.5), with probability level of 0.1129 (Table 2), so showing non-significant difference. Fishman (1980) reported that a mild anaemia was often present. It resembled the anemia of other chronic infections but was usually less severe.

Total leucocyte count (TLC): Total leucocyte count of normal subjects was 6213.4 ± 1161.68 (Table 2), while of Tb cases was 8882±1650 (Table 2) with probability value 0.000, expressing highly significant difference of TLC between Tb patients and control subjects. Fishman (1980) reported that TLC in peripheral blood was often normal and rarely over 15000/cmm. Over 20,000/cmm suggested some infectious process. High ESR values and high leucocyte count in case of pulmonary Tb was narrated by Miller *et al.*, 1989; Wislowska, 1989; Niikawa *et al.*, 1988. When data were analyzed on the bases of different age groups (Table 3 and 4), TLC of age group 1(12-22) of control subjects was 6090.71 ± 1348.00 (Table 3) while, in Tb patients (9182.72 ± 1686.86) so, highly significant difference was present (Table 4). In age group II (23-32) of control subjects, TLC was (6658.33 ± 999.58) while in Tb cases (8008.33 ± 1778.38), (P<0.0318) showing significant difference. In age Group III (33-42) of control subject has TLC (6350.00 ± 1143.67) while, Tb cases had (8085.71 ± 2152.65), P<0.015 expressing non significant difference. In age Group IV (43-52) of control subjects the TLC was (6462 ± 1506.82), while of Tb case was (9025 ± 989.94) with (P<0.0013) showing significant difference of values. In age group V (53-62) of control case TLC was (5820 ± 739.59), while of Tb cases was (7540 ± 1052.6) with probability level (P<0.0173) expressing significant difference. In age group VI (63-72 years) the TLC values in normal subjects was (5550.0 ± 360.55) while in Tb Cases was (8066.6 ± 1592.06) with (P<0.0045) so, expressing highly significant difference of values in both Tb cases and control cases. From the data, it was concluded that only age group (33-42 years) showed non significant difference of TLC values of normal and diseased persons, but in most of age group there was a significant increase of TLC in Tb patients as compared to control subjects (Table 3 and 4).

When sex based analysis of TLC was made, there was a non-significant increase (P<0.120) of TLC in control male subjects (6382.4 ± 1108.26) than female control subjects (5952.94 ± 1238.0). Same case was present in Tb males (8470.31 ± 1648.2) and females (8225.55 ± 1689.8), with probability level (P<0.111) so, non-significant difference was present (Table 2).

Differential leucocyte count (DLC): The neutrophil count of control subjects was (60.04 ± 5.15), lymphocyte count (32.39 ± 4.63) eosinophil count (3.2 ± 1.55). Monocyte count (4.38 ± 2.55) and basophil count was (0.68 ± 0.27) (Table 2). While the Tb subject had neutrophil count (69.4 ± 4.62), lymphocyte count (24.55 ± 3.6), eosinpil count (2.16 ± 1.34), monocyte count (3.7 ± 1.72) and basophil count (0.08 ± 0.27) (Table 2).

Statistical analysis of DLC values of normal and Tb cases yielded such as neutrophil: showed significant difference with P<0.000 eosinophil showed significant difference with (P<0.0004), while monocyte showed non-significant difference (P<0.1127) and basophil non-significant difference (P<1.00) (Table 2).

Fishman (1980) demonstrated, that differential white blood cell count was usually normal except when the tuberculosis disease was advanced and active. Although, changes did occur in relative numbers of lymphocyte, monocyte and polymorphonuclear leucocyte, these had not proved useful either as clinical or prognostic indexes. According to the findings of Onwubalil and Scott (1988), during Tb there were a series of metabolic and immunologic abnormalities, including evidence of under nutrition, anaemia, neutrophil leucocytosis, monocytosis, lymphopenia, hyperglobulinemia and raised ESR. A comparative study of Robles and Reyes (1994) revealed leukocytosis, neutrophilia, high ESR and high fibrinogen in case of tuberculosis. Age based data revealed the different DLC values in age group I(12-22) of control persons is neutrophil (57.5 ± 7.01), lymphocyte (34.14 ± 5.9), eosinophil (3.07 ± 1.89), monocyte (4.78 ± 2.69) and basophil (0.14 ± 0.36) (Table 3). While in Tb cases, neutrophil (68.54 ± 4.16), lymphocyte (25.45 ± 2.94), eosinophil (2.09 ± 1.22), monocyte (2.90 ± 1.45) (Table 4).

Data showed non significant difference of DLC on the basis of sex in normal cases (Table 2). From the analysis of data (sex based) it was clear that there was unclear differentiation of DLC values in male and female Tb patients (Table 2).

Analytical epidemiology: Analytical epidemiology study was conducted on three groups, divided on the basis of age as youngsters (unmarried) including males and females of age below 24 years, males (married) and females (married).

On the basis of detailed analytical epidemiogical data, it came to know that many of village dwellers were using raw milk. Collier *et al.* (1998) discovered that animal reservoirs of *M. bovis* posed a serious threat to human health. Tb in cattle principally involved the lung and it spread from animal to animal by cough spray. Humans in direct contact with cattle might like wise be infected and developed primary pulmonary lesions. Milk was the principal vector of transmission of *M. bovis* to town dwellers. Such transmission was facilitated by the pooling of milk from many cows and herds. The analysis of data also showed that many patients were having chains of Tb patients at home and one person mostly developed Tb, from previously existing Tb source. These findings, also supported their lack of knowledge about prevention from the disease. Risk for children existed in conditions of contact with subjects with marked residual post-tuberculosis alteration (Grishko and Vasilev, 1995). Korablev *et al.* (1995) stated that contact with Tb patients was responsible for new cases, discharging drug resistant MTb. Family members and neighbours of patients in active Tb of lungs were at great risk, to develop Tb than other population (Samuservich, 1995). Forty seven percent (47%) of total Tb patients were misdiagnosed and received incomplete treatment. According to the studies elaborated by Kovalev and Gvozdilkin (1994) the tuberculosis had a risen by chance as a result of misdiagnosis and poor control over persons to be treated.

Mass media should play an important role in bringing awareness among the people about curing the disease sooner its symptoms occurred. Previous admission to hospital, failure to isolate positive patients in a single room and the absence of positive pressure were associated with spread of Tb. Control measures could halt the transmission of MDR strains (Collier *et al.*, 1998). 82% of admitted Tb patients were smokers among males. Collier *et al.* (1998) demonstrated the higher incidence of Tb in men, who smoked and drunk exclusively. Hospital records were reviewed by Pratl *et al.* (1980) to ascertain smoking history and occupation. Data showed significant effects of smoking on the disease. Bronchitis and Bronchiolitis were associated with textile industry. A significant association was found between cotton dust exposure and both mucus gland hyperplasia.

The comparative efficacy of different tests used in the study can be inferred from the findings that Mantoux test had no correlation with hematological values and the AFB test was limited by the size of the sample. In AFB test positive cases are detectable if the bacterial count in the sample is about 10^4/ml. The detection of Tb through X-ray is not reliable in small children. The hematological

values in all tests were found to be affected by the age and sex of the population. These values were found to have no consistent correlation with any method used in this study. PCR technique was found to be more sensitive, specific and a tool to confirm Tb qualitatively and quantitatively and thus being the only reliable technique is worthy of use in reliable diagnosis of Tb in human beings.

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