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ANTI-TUBERCULOSIS ASSAY FOR MOLECULAR EPIDEMIOLOGY OF MULTI DRUG AND EXTENDED DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS IN AN URBAN SETTING

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Abstract

Objective This study was aimed to detect the rate of positive cases in urban setting of Punjab along with MDR and XDR cases using LPA, GeneXpert and hematological profile.

Design: A cross-sectional study.

Setting: The study was conducted at the Punjab Aids Control Program. All sample were collected along with the socio-demographics characteristics of patients.

Main Outcome Measure: These samples undergo Microscopy, Culture, Gene X pert and LPA to confirmed microbiological status of patients. EDTA collected blood was tested for complete blood counts using hematology analyzer.

Results Out of 1335 positive samples confirmed by Culture, Microscopy, GeneXpert and LPA, 4.5% were resistant to rifampin and isoniazid while 3.5% were resistant to second line fluoroquinolones using LPA. 14.7% Neutropenia, 17% anemia and 5.54% thrombocytopenia were observed. ESR was high in all participants.

Conclusions: GeneXpert and LPA to be a fundamental diagnosis for the detection of MDR and XDR. LPA outperformed in detection of resistant of TB compared to GeneXpert. It is better than conventional culture in terms of resistance detection. Prevalence of anemia and neutropenia was high in patients with TB and other co-morbidity.

Keywords: *Mycobacterium tuberculosis,* Line probe assays (LPA), Gene X pert, rifampin, Resistance, fluoroquinolones.

1. Introduction

Mycobacterium tuberculosis (MTB) is one of the leading causes of death among infectious disease [1]. According to latest report of World Health Organization (WHO) about 45 million deaths occur annually which includes 15 million individual that are co infected with HIV and 104 million positive cases are reported each year particularly in developing countries such as Pakistan, Bangladesh etc. The eradication of TB from world could achieved by joint efforts of international health organizations upto the year 2050 [2].

TB is transmitted through respiratory droplets produced by infected person during speaking, coughing, sneezing and singing and then inhale by healthy person [3]. There are certain vital risk factors that play an important role in complication and exaggeration of TB that involves HIV co infection, Diabetes, Hypertension, Cancer, Liver and kidney diseases, post-surgical suppression, drug abuse and smoking [3]. Once *Mycobacterium* enters the body reactive oxygen species formed as a result of activation of phagocytosis by immune system. These reactive species try to kills MTB but in turn harms the host cells and caused inflammation and injury [4].

There are some challenges that need to be address such as multi drug resistant (MDR), extended drug resistant (XDR) TB and co infection with other microbes such as Human immune deficiency virus (HIV) that requires long course of treatment, Care and increase economic burden as well. MDR and XDR increase mortality rate [5-9].

For sensitive TB there is a six months course of treatment which include drug that contain certain active agents Rifampin, Isoniazid, ethambutol and pyrazinamide for first 2 months and then next four months pyrazinamide is excluded [10, 11]. MDR TB strains are those which are resistant to at least two first line agent such as rifampin and isoniazid. XDR MTB strains are those which undergo such a mechanism of resistant so that first line agents along with second line fluoroquinolones does not works on them and require such agents for treatment that are more toxic and require long course for adherence of treatment [12].

Totally drug resistant (TDR) TB is worse and drastic of all because in that case all first line and second line drugs of TB are resistant to that strain. It was first reported from Italy in 2007 and then from Iran, India and South Africa. To cure such cases certain agents were introduced by WHO those are high dose of isoniazid, Thioacetazone, Linezolid, Macrolides and Carbapenem but there efficacy, toxicity and safety is still questioned [13, 14].

Culture is gold standard for diagnosis of TB but require long incubation that results in delay of treatment. Microscopy has high specificity for diagnosis purpose in high prevalence areas but unable to distinguish between live and dead bacilli [15]. GeneXpert is latest molecular test for diagnosis of TB in which DNA and rifampin resistant gene could be detected in a single tube, for shorter time with efficiency and MDR cases could be separated. Its turn-around time (TAT) is 2 hours [16]. In conventional method of sensitivity require culturing that could require special media and long-time of incubation along with 5%-10% of CO_2. Then sensitivity is performed on special media, long period of incubation, care and follow up that is burdensome and may results in increase mortality due to delay in results and false reports due to contamination [17].

Many molecular level methods are available to detect sensitivity of drugs against strains of TB such as Loop Mediated Isothermal Amplification (LAMP), Line Probe Assays (LPA), Gene X pert, Whole Genome Sequencing (WGS) and MTBDR Plus [18]. Line probe assay and Gene X pert was approved by WHO to be applicable in public setting to spot cases of MDR and decrease economic loss in 2008 and 2010 respectively [19]. Certain gene mutations against anti-tuberculosis drugs have been reported such as *rpoB, KatG, rrs, inhA, rpsL, gyrA, embCAB* and *panA* [20]. This study is aimed to detect the rate of positive cases in urban setting of Punjab along with MDR and XDR cases using LPA, Gene X pert and hematological profile.

2. Materials and Methods

2.1. Study Area

Lahore is a populous city of Punjab Pakistan. This was a prospective study enrolling 1352 samples which were based on information of clinical presentations and microbiological data of patients who were tested for diagnosis of TB along with resistant using culture, Microscopy, X-ray LPA and GeneXpert from 2017 to 2019. The population of the study included patients who were referred to the Provisional Reference Laboratory (PRL) Primary and Secondary Healthcare and Punjab Aids control program (PACP) from different hospitals of Lahore such as Ganga Ram hospital, Jinnah hospital, Munshi hospital, and Children hospital etc.

2.2. Study Subjects

This study includes the participants that are suspected for TB due to clinical manifestations that includes coughing more than two weeks, Weight loss, Loss of appetite, Night sweating, Hemoptysis and Malaise. Both Gender and all age groups were included. Demographics include their Profession and Income status also consider in it.

2.3. Collection and diagnosis of samples

Sputum sample were collected in sterile and open mouth container in hospitals and transported to the laboratory at 4^{o}C and following test are performed for diagnosis of TB within 24 hours. For confirmation in addition to clinical signs and symptoms basic test were performed on all samples that includes Microscopy of Acid-fast bacilli using Zeil and Nelson stain and bacterial load is also reported as +, ++, +++, ++++. Culturing was done after decontamination with NaOH on Lowenstein Jensen (L-J) medium under aerobic conditions at 37^{o}C for 6 weeks. GeneXpert is latest, rapid and easy to use and is determined Rifampin resistant gene along with bacterial DNA. Line Probe assays (LPA) a DNA strip method that detects the mutations in genes that results in resistant to most important drugs against TB. Radiological examination is based on the X-Ray.

2.4. Data analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for window version 23. Frequency and percentages were calculated. Chi square test and correlation was used to determine the relationship between variables.

2.5. Patient and Public Involvement:

This is retrospective data base study. So, no patient is involved directly.

3. Results

During the study period of 2 years, from the total of 1352 samples that were collected and processed, the assay provided valid results in 1335 (98.7%) samples and unsuccessful results in 17 (1.2%) samples. Hence, 1335 samples were the part of the study. The incidence of tuberculosis was 0.01% among which 48.5% were male and 44.3% were females while females were more resistant to males. 4.5% were resistant to rifampin and 3.5% were even resistant to second line drug fluoroquinolones and incidence rate was high in females (52.5%) than males (47.5%). Most of the resistant candidates were from laborer and addictors (14.7%) followed by retired (13.1%) and belong to lower middle class (59%).

The socio-demographic aspect of the samples gave results of a mean age (range) of 45 (3-92) years and median and mode of 48 and 56 years respectively. Most of candidates included in the study were Adults (88.8%) and males (52.3%) with professional status of lower middle class. Table no 1 shows frequency and percentages of all variables involves in our study.

Table no.1 Demographics Data of Respondents

	FREQUENCY	**PERCENT (%)**
GENDER		
FEMALES	645	44.3
MALES	707	48.5
OCCUPATIONS		
ADDICTOR	102	7.0
TEACHER	170	11.7
HOUSEWIFE	126	8.6
FACTORY WORKER	173	11.9
HEALTH CARE WORKER	76	5.2
ENGINEER	125	8.6
IT WORKER	90	6.2
STUDENT	106	7.3
RETIRED	155	10.6
NULL	3	0.1
SALESMAN	42	2.9
LABORER	185	13.85
STATUS		
MIDDLE CLASS	592	40.6
LOWER MIDDLE CLASS	759	52.1
AGE		
<20 YEARS	133	9.1
20-40 YEARS	459	31.5
41-60 EARS	479	32.8
>60 YEARS	280	19.2
CO-MORBIDITIES		
ADVANCE AGE	37	2.7
HYPERTENSION	351	25.9
DIABETES	178	13.1
CANCER	23	1.7
LIVER DISEASE	55	4.0
POSITIVE TB STATUS		
TB	1291	95.4
MDR	61	4.5
XDR	46	3.5

3.1. Outcomes of GeneXpert along with Microscopy

The GeneXpert assay test for the detection of MTB was positive in 1328 (99.4%) samples and negative in 7 (0.6%) samples; 94.6% of the positive samples were with positive Z-N staining and 5.4% of the positive GeneXpert MTB assay tests were negative on Z-N staining. Table no 2 showing baseline of TB along with their resistant status.

Table no.2 Baseline of TB Along with Their Resistant Status

	FREQUENCY	PERCENT (%)
TB RELATED TEST		
X-RAY	1457	100
MICROSCOPY	1457	100
CULTURE	1325	90.9
GENE X PERT	1327	91.1
LPA	164	11.3
DRUGS		
FIRST LINE AGENTS		
RIFAMPIN	61	4.1
IAONIAZID	52	3.5
SECOND LINE AGENT		
FLOUROQUINOLONES	48	3.2
KANAMYCIN	1	0.06
AMIKACIN	3	0.2
KANAMYCIN / AMIKACIN	3	0.2
LOW LEVEL KANAMYCIN	1	0.06

3.2. Hematological profile indicator of severe Tuberculosis

Complete blood count is considered baseline along with ESR to confirm inflammation in TB. It includes many parameters such as Hb, HCT, MCV, MCH, MCHC, Red blood cells (RBC's), White blood cells (WBC's), lymphocytes and Platelets. 17% cases of Confirmed tuberculosis by Microscopy, Culture, GeneXpert and LPA shown anemia in patient among which most of were suffering from Microcytic anemia. Neutropenia (14.7%) and thrombocytopenia (5.54%) were also observed in patient with co morbidities. The mean and median of Hb along with MCV is 12.5, 12.9, 85.3 and 86 respectively. ESR was high in all patients. Table no 3 has shown mean, mode, median, range and standard deviation of Baseline in combination with ESR and Age.

Table no.3 Baseline Parameters in Combination with ESR and Age

CBC	Mean	Median	Mode	Standard deviation	Range	Minimum	Maximum
Hb	12.59	12.9	14	2.15	17	3.2	17
RBC's	4.6	4.6	5	0.79	8	1	9
HCT	39.21	40	40	6.43	48	17	65
MCV	85.74	86	85	7.98	74	56	130
MCH	27.75	28.0	26	3.30	34	13	47
Platelets	270	267	295	90.25	436	62	498
ESR	111.9	115	120	27.0	130	55	185
Eosinophils	2.6	2	2	1.9	11	0	11
Monocytes	5.82	6	6	3.1	43	1	44
Neutrophils	57.2	60.0	58	22.6	96	0	96
WBC's	8.49	8.4	6	3.1	18	1	19
Lymphocytes	25.96	28	21	19.7	374	0	374
MCHC	32.3	32.9	32	1.90	18	21	39
Age	45	48	56	17.7	89	3	92

3.3. Epidemiology of rifampicin-resistant and fluoroquinolones-resistant Mycobacterium tuberculosis and characteristics of samples

Figure 1 shows the graphical representation of resistance tuberculosis. While conducting research out of 1335 samples 61 were Rifampin resistant which were detected on Gene X pert followed by LPA by detecting the mutations. Among all Gene X pert positive cases (4.5%) were of rifampin

resistant. Using LPA gene mutations which were responsible for resistant of fluoroquinolones were 3.5%. One case is reported as Total drug resistant (TDR) that means Strain is resistant to all first- and second-line agents. Resistance was observed primarily in females and adult age group within addictors and Retired persons. Lower middle class (59%) is more prone to resistant cases. Education status of resistance samples was mostly illiterate with employment status of retired, laborer and addictors (13%), (14.75%) and (14.75%) respectively. 9.8% cases of MDR were reported with co-morbidities that include advance age and hypertension most. Among 48 cases of XDR males (58.3%) were more resistant compared to females. Persons with advance age and co-morbidities were more resistant to second line agents which would results in mortality. Table no.4 has shown the demographic data in relation with rifampicin-resistant and fluoroquinolones-resistant Mycobacterium tuberculosis.

Table no.4 Demographic Data in Relation with Rifampicin-resistant and fluoroquinolones-resistant Mycobacterium tuberculosis

	Rifampin sensitive		**Rifampin resistant**		**fluoroquinolones resistant**		**Chi-square**	**df**	**P value**
	1291	95.4%	61	4.5%	48	3.5%			
GENDER									
FEMALES	604	44.6	32	52.4	21	43.75	683-2730	18	.000
MALES	665	55.4	29	47.5	27	56.25			
OCCUPATIONS									
ADDICTOR	93	7.2	9	14.7	6	12.5	751-2782	78	.000
TEACHER	161	12.47	7	11.4	4	8.3			
HOUSEWIFE	122	9.45	4	6.55	3	6.25			
FACTORY WORKER	169	13	3	4.9	2	4.1			
HEALTH CARE WORKER	72	5.5	4	6.55	4	8.3			
ENGINEER	116	8.9	3	4.9	6	12.5			
IT WORKER	84	6.5	4	6.55	4	8.3			
STUDENT	98	7.5	6	9.8	4	8.3			
RETIRED	145	11.23	8	13.1	5	10.4			
NULL	2	0.1	0	0	0	0			
SALESMAN	38	2.94	4	6.55	3	6.25			
LABORER	169	13	9	14.7	7	14.5			
STATUS									
MIDDLE CLASS	556	43	25	40.9	20	41.6	675-2729	18	.000
Lower Middle Class	713	55.2	36	59.	28	58.3			
CO MORBIDITIES									
ADVANCE AGE	30	2.3	6	9.8	5	10.41	184-1593	36	.000
CANCER	22	1.70	1	1.6	2	4.16			
HYPERTENSION	347	26.8	6	9.8	3	6.25			
DIABETES	171	13.2	3	4.91	1	2.0			
LIVER DISEASE	53	4.10	2	3.27	1	2.0			

4. Discussion

Transmission of tuberculosis from infectious source could depend on the air, light, immune status of persons and duration of exposure [21]. This cross-sectional study had selected the data of socio-demographic and epidemiology of MTB and RR-TB identification by Z-N staining, GeneXpert MTB and LPA assays. GeneXpert MTB and LPA assays was selected due to feasibility of healthcare facilities in relation with previous history, sign & symptoms and resistant status of TB to ensure the correct treatment in timely manner [22]. GeneXpert was recommended, reliable, accurate and rapid technique for the detection of TB approved by (WHO) in 2010 [23].

Our results also shown that tuberculosis may develop anemia in subjects and vice versa with co morbidities as 17% of individual were anemic with low Hb, MCH, MCHC and MCV. A systematic review was performed in 2021 shown that anemia results in decrease immunity thus individual become susceptible to infectious disease including tuberculosis [24]. Our study shows that

neutropenia is 14.7% and thrombocytopenia is 5.5% in tuberculosis suffering patient along with co morbidities. A study conducted in Ethiopia in 2019 supports our finding that anemia and neutropenia is more in TB patient suffering along with co morbidity such as HIV [25].

A study conducted 3 years ago in Karachi Pakistan shows that only 22.4% women were screened for TB and get their sputum checked while males were 77.6% [26]. Tuberculosis is considered stigma in women because men get more attention and care during treatment. Man are more susceptible to Tuberculosis due to their habit of smoking that makes their lungs weak and prone to *Mycobacterium* [27]. Our study also shows similar finding that females are dominated by males in screening of TB as 44.3% of females got screen and diagnose only rather than males which are 48.7%. A study conducted in 2021 and their results indicate that Un-employed person are more prone to developing the Tuberculosis compared to employ and most common co-morbidity is hypertension that could worsen the situation by FA Mphande Nyasulu and his colleague [28].

This study strongly favors our results as retired and adductors are more at risks to develop tuberculosis. Most common co morbidity that impacts our study population is hypertension (25.98%) being at top of co morbidities and others is as follows diabetes (13.1%), Liver disease (4.0%), advance age (2.7%) and cancer (1.7%) respectively. In past HIV co infected with TB consider the difficult case to handle but now diabetes even cross that limits and results in reactivation of tuberculosis. Diabetes could result in increases levels of pro-inflammatory cytokines and decrease in levels of anti-inflammatory cytokines which could leads to inflammation and make TB control difficult. It could results in Resistant due to constant exposure of anti-tuberculosis drugs [29].

Occupation and education level has great impacts on the TB status prove by different studies. Occupation level leads to experienced and knowledge and ultimately knowledge leads to prevention [30]. A review used three different mechanisms to see the impact of occupation in development of tuberculosis. Third mechanism prove that laborer, prisoners, homeless, retired and healthcare worker develop tuberculosis more compared to people working in a dustless environment [31]. Our study shows that people with poor knowledge being laborer (13.6%) suffer more from tuberculosis than other professions and those who works in areas where dust is more, light and air could not pass frequently such as factories and basements so their employ are more at risk of developing TB.

According to our research Income status shows that lower middle class suffer more compared to middle class due to their houses, life styling, diet and immune status. Less knowledge regarding TB may be a factor in increases incidence of TB. A study conducted by Shailly and his colleague in 2019 that shows incidence of TB is as follows; in upper lower class (84%), lower middle class (11%) and upper middle class (4%). So, by this study lower class has highest risk of TB as similar in our research. It is due to inadequate intake of food, less proteins and calories in diet effects your immune system and results in Tuberculosis [32].

Our study shows that our 95.5% participants were sensitive to first line agents of tuberculosis and follows normal course of treatment. While on other hand 4.5% were resistant to rifampin or either isoniazid being MDR and 3.5% were resistant to fluoroquinolones and results in the XDR thus increases economic burden, require long course of treatment. A study also support our finding that multi drug resistant cases were up-to 4.3% and its burden is increasing day by day almost 62% burden was reported worldwide (China, India and Russia) by Matteo Zignol in 2005 [33].

We also found a case during our research in which all first line agents and second line agents are resistant to infectious strains of tuberculosis and then patient used high dose of isoniazid, linezolid, carbapenem etc. that has poor efficacy and high toxicity to patient. MDR and XDR could be treated to some extent but total drug resistant pose a threat to humanity and undergo such mutation that results in severe mutation [34].

Extensive drug cases are resistant to second line injectable and fluoroquinolones and reported 3.4% by previously conducted study in Ethiopia and pre-extensive drug resistant was about to 5.7%. They may be resistant to low level kanamycin [35]. It has been found that multi drug resistant is associated with previously treated infection with tuberculosis, co infection with HIV, co morbidity

such as diabetes and hypertension and Less BMI. It mostly affects working group due to their wide exposures and affect economy [36].

We used the technique of Gene X pert and LPA for detection of mutation that results in resistant LPA is expensive and require expertise to perform but has the ability to detect the mutations that results in mutation of any of four first line agents and all second line agents too. Gene X pert is cheap and simple but is able to detect only gene that results in rifampin mutations and differentiate as Simple TB and MDR TB. Diagnosis of tuberculosis should be done by combination of test using smear, culture, Gene X pert and X ray. Molecular DST should be reported using LPA to ensure correct treatment, prevention and prognosis [37].

Strength and Limitations:

- This is perhaps among the very few recent studies aiming to explore the incidence of TB in the region and the factors associated with its prevalence.
- This study has provided a new insight about for the future research to explore success of the pharmacotherapy programs that are functioning in Pakistan
- Our reliance was on the data base only and it is possible that if this study was planned face to face with patient more in-depth exploration of the factors associated with the disease and its outcomes can be explored

5. Conclusions

Mycobacterium tuberculosis is a public threat to lower income countries and require to stop in order to reduced economic burden. Increasing incidence of Multi drug resistant Tuberculosis and extended drug tuberculosis require a rapid method because diagnosis play a key role in treatment, prevention, and stop of spread of tuberculosis and helps in reducing economic burden and decrease mortality. LPA is a molecular technique for detection of tuberculosis resistant mutations in MDR and XDR. Gene X pert is cheap and easy to perform and able to spot the resistant cases against first line drug rifampin in only two hours. This could help public health organization to plan such strategies to diagnose resistant cases and isolate them so reduced spread of resistant cases. It could help to reduced mortality and economic burden.

6. Competing Interest

The authors declare that they have no competing interests.

7. Funding:

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

8. Data Sharing Statement:

No Additional Data Available.

9. Patient Consent:

Not Applicable

10. Ethical Approval Statement:

The study protocol was approved by the institutional review board of the University of the Veterinary and Animal Sciences Lahore (Ref: DAS/329/30/5/2017).

11. Figure Legend:

Figure 1 shows the graphical representation of resistance tuberculosis. Out of 1335 samples 61 were Rifampin resistant which were detected on Gene X pert followed by isoniazid and fluoroquinolones.

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