Pakistan J. Zool., pp 1-12, 2026. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20250703171631

A Study on the Prevalence and Molecular Confirmation of *Pulmonary tuberculosis* Using GeneXpert MTB/RIF and its Comparison with Polymerase Chain Reaction and Conventional Assays Among the Outdoor Comorbid Isolates from Quetta, Pakistan

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Article Information
Received 03 July 2025
Revised 20 August 2025
Accepted 30 August 2025
Available online 29 January 2026 (early access)

Authors' Contribution
AK: Conceptualization, design, investigation, writing-original draft and supervision. MY: Review, conceptualization, design. MR, MD, MH and MS: Contributed equally in reviewing editing and analyzing. All authors critically reviewed the manuscript and approved the final version of the manuscript.

Key words
Pulmonary tuberculosis (PTB); Comorbid suspects; GeneXpert MTB/RIF; Lowenstein–Jensen medium (L.J); Ziehl–Neelsen staining (Z.N); Acid fast bacilli (AFB); Quantitative Polymerase chain reaction (qPCR)

ABSTRACT

Tuberculosis (TB) is a major global public health concern and every year millions of people are affected and dies from it, particularly in developing countries. Yet, there is no data available about pulmonary TB in comorbid isolates of Quetta, Pakistan. This study aimed to determine the incidence and related risk factors of pulmonary TB in comorbid suspects using the molecular GeneXpert MTB/RIF assay and compare its results with quantitative PCR assay and traditional diagnosing methods. A total of 700 TB suspects were included in the study, in which more females were effected (59%) than male (41%) and married couples were more observed among the majority of suspects. The majority of suspects were 37.71% for around 46 to 60 years of age and followed by (30%) suspects having age between 31 to 45 years (P>0.05). Among the suspects, medical comorbidities like asthma, diabetes (DM), hypertension, and hepatitis were observed (37%, 28.9%, 21%, 11% respectively) (P > 0.05). Nevertheless, a statistically significant association was recognized in variables such as asthma. In this study, weakness and fever was noted as the most common clinical sign (72%), followed by anorexia, chest pain, weight loss, night sweat, and cough (62%, 58.1%, 55%, 54% and 45% respectively), among the suspected comorbid isolates. Significant association was identified in variables such as night sweat, (P = 0.030). In the suspected sputum samples, 12.71% were PTB-SS positive, whereas the remaining were PTB-SS negative. Further, the sputum samples showed 17.43% positive growth rate (MTB colonies). In addition, 95.08% of the comorbid suspect's sputum samples were confirmed using molecular Xpert assay. However, qPCR assay sensitivity was 99.18%. Overall, the prevalence of pulmonary TB was highest in comorbid suspects and GeneXpert MTB/RIF assay is an innovative molecular assay, its sensitivity was higher in comorbid isolates for the detection of pulmonary *M. tuberculosis* and Rif resistance, suggesting the spread of pulmonary TB to healthy humans. Therefore, prompt, regular treatment and effective procedures are needed to prevent the transmission of active TB cases in the comorbid population of the present study area.

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0030-9923/2026/0001-0001 $ 9.00/0

INTRODUCTION

Tuberculosis (TB) is the most chronic and contagious disease and causes high mortality in the developing countries. However, it is curable and preventable (Kalonji *et al*., 2016; Khan *et al*., 2020). It is recognized globally as a major cause of morbidity as well as mortality among the infectious diseases after human immunodeficiency virus

(Shafee *et al*., 2019; Khan *et al*., 2020). In most developing nations, many comorbid risk factors and diseases including, diabetes, asthma, smoking, undernourishment, depression, malaria, hepatitis, HIV and chronic lung disease, etc. double the burden of diseases like TB (Bates *et al*., 2015; Al-Salihi and Mankhi, 2020; Shrestha *et al*., 2020; Alemu *et al*., 2021). Transmission of this infectious disease generally occurs only when someone is in considerable contact with active TB patient. Spread of the initial TB infection occurs in a year and results in primary disease. However, it can also stay dormant but remain viable for years. If the immune system of TB-infected human is weak or compromised, bacteria may be re-activated and cause active TB (Lienhardt *et al*., 2012; Sanusi *et al*., 2017).

Typically, in diagnosing methods the most traditional *Mycobacterium tuberculosis* (MTB) detecting assay is Ziehl-Neelsen (ZN) staining. Although it is less sensitive and specific for detecting MTB tubercle bacilli. However, it is gold standard diagnosing tool and mostly used (Singhal and Myneedu, 2015; Bajrami *et al*., 2016; Khan *et al*., 2020). Similarly, culture methods like Löwenstein–Jensen (LJ) and M.O, are more sensitive and gold standard diagnosing methods but have limitations due to its long time consumption (6-8 weeks), form initial culturing till to final results for MTB interpretation, requirements of biosafety laboratory (BSL-III and BSL IV) and high cost requirements (Palaci *et al*., 2013; Uddin *et al*., 2013; Organization, 2015; Singhal and Myneedu, 2015; Ahmed *et al*., 2019). Polymerase chain reaction (PCR), an advance molecular diagnosis approach which are more sensitive and reliable laboratory TB diagnostic tool (MacLean *et al*., 2020; Sharma *et al*., 2023). Although, it requires technical skills and cost- effective and used in detection of MTB due to its higher sensitivity and time reduction (Luukinen *et al*., 2019).

Similarly, GeneXpert MTB/RIF assay is an advance, automated, computer linked, rapid and real-time PCR based MTB and rifampicin resistance detecting assay (MTB/RIF). It is most convenient over traditional diagnosing assay (sputum smear and culture procedures). Furthermore, it detects rifampicin-resistant (RIF) tubercle bacilli within 2 h (Organization, 2015; Bajrami *et al*., 2016; Dorman *et al*., 2018; Soeroto *et al*., 2019). GeneXpert MTB/RIF assay amplifies the *rpo*B gene (MTB complex-specific region), probed through molecular beacons to sense the presence/absence of RIF, determining mutations in MTB genome (Soeroto *et al*., 2019; Dorman *et al*., 2018). However, there are very rare GeneXpert MTB/RIF based molecular studies in comorbid isolates in the current study area.

Recent reports by WHO has shown around 10.8 million infections and 1.4 million deaths occur globally due to TB (Grobusch and Kapata, 2018). Further, studies have shown that approximately 510 000 cases (270 per 100000 population) of TB are reported in Pakistan (Grobusch and Kapata, 2018; Shafee *et al*., 2019). At present, 22 high TB burden nations accounts for more than 80% of world's TB cases and Pakistan is on the fifth position among high TB infected countries (Ahmad *et al*., 2018).

In addition, Baluchistan is the largest province of Pakistan by area and very limited data is available regarding the incidence of TB (Niamatullah *et al*., 2018; Khan *et al*., 2020). Similarly, it has lowest per capita income as well as literacy rate than the other areas of the country. Quetta, the capital of the province with approximately, 2.8 million populations and its borders being connected with Afghanistan in the north- East (war effected) as well as with Iran in south (U.S. sanctions) and also harbors above 0.3 million refugees (Shafee *et al*., 2019). Further, most of the people are living in combined family system and those living in far rural areas have poorer asses to hospitals for diagnosis. Lower education rate, social stigma and lack of awareness to this infectious disease have further worsen the situation (Getnet *et al*., 2017; Kakar *et al*., 2018; Shafee *et al*., 2019; Ereso *et al*., 2020). Recently media reports have shown more than 25,000 TB cases are emerging annually in Balochistan. Further, previously no research data is available about the prevalence of TB in comorbid isolates in the populates of Quetta, Pakistan. Therefore, this study was conducted to evaluate the prevalence of tuberculosis in the outdoor comorbid isolates using GeneXpert MTB/RIF, an advance molecular tool, with other traditional diagnosing methods (L.J culture and AFB Z.N staining) and further compare it sensitivity with PCR for diagnosing and determining the incidence of TB in outdoor comorbid patients.

MATERIALS AND METHODS

Study design

A total of 700 suspected with many comorbidities (for example, hepatitis, asthma, kidney or heart diseases) and along with history of persistent cough, low-grade fever, loss of appetite and chest pain were included in this study. Demographic and clinical signs and symptoms data of suspects were noted including age, gender, literacy rate, and continued cough, low-grade fever and chest pain. In addition, a random pulmonary sampling technique was used for all TB suspects aged between 15 to $\geq$ 50 years. Sputum samples were collected to investigate TB bacilli using Ziehl-Neelsen (ZN) smear microscopy and all sputum samples were inoculated on LJ medium to confirm MTB bacilli in the individual's sputum samples. The positive sputum samples of the TB suspects that were confirm through LJ method were also processed for GeneXpert and qPCR assay. Sputum samples after initial

screening were processed and kept at -20°C for further usage.

Sputum collection and ZN smear preparation for AFB

Sputum samples were collected from all suspected patients in sterile container and sputum smear were prepared as proposed by (Shafee *et al*., 2014; Khan *et al*., 2020).

LJ medium is an egg-based medium and is more specific for growth of MTB (Kent, 1985). Sputum specimens were decontaminated using NALC-NaOH procedure (Khan *et al*., 2020). In brief, a fresh malachite green solution (2%) and whole egg homogenate was prepared. Subsequent to decontamination of specimen (NALC-NaOH procedure), inoculation of the specimen was done in LJ slants, and incubated at 37°C up to 8 weeks. After 48 h of incubation, the growth was confirmed by the morphology of colonies, that is, rough, buff and tough MTB colonies, no pigment production in inoculated media bottles, slow growth rate of colonies incubated at 37°C, and slightly straight or cured, red colored AFB smear as observed through ZN staining and biochemical tests (Niacin production and nitrate reductase activity) as performed by Kent (1985).

GeneXpert MTB/RIF assay of sputum samples

Sputum sample for GeneXpert MTB/RIF assay were prepared as previously reported by Dorman *et al*. (2018), and Soeroto *et al*. (2019). Briefly, the MTB/RIF sample reagent (Cepheid) and with 1 ml of sputum was mixed (2:1 ratio) and homogenized with vortex. Then 2ml of homogenized sputum mixture was transferred into cartridge (Xpert MTB/RIF assay) and inserted it into GeneXpert MTB/RIF tool (Boehme *et al*., 2010; Soeroto *et al*., 2019).

Quantitative PCR assay for amplification of target MTB genes

M. tuberculosis DNA was extracted according to Chaoui *et al*. (2009), and Islam *et al*. (2020), with some modifications. Briefly, scraped *M. tuberculosis* colonies from L-J culture were re-suspended in (100-200 µL) of distilled water and boiled it at 100 °C for 10 min to inactivate and release the MTB DNA. Subsequently, centrifuged it at 10000 rpm for 10 min, the supernatant comprising the genomic DNA was directly used for PCR amplification or stored at −20 °C for later use.

Quantitative PCR was used to confirm the isolated MTB isolates through GeneXpert based assay and rifampin resistant MTB isolates as described in the previous studies (Eisenach *et al*., 1990; Yam *et al*. 2004; Yang *et al*. 2005; Singh *et al*. 2016; Sah *et al*. 2017; Kakar *et al*. 2018; Salim *et al*., 2021). The targeted genes were; *IS6110* gene and *rpo*B gene. PCR primers for target genes are shown in (Table I). For each qPCR reaction, MTB DNA (H37Rv strain) and PCR grade water were used as positive and negative controls. All PCR amplicons were separated on 2% agarose gel electrophoresis and stained with (0.5 µg/µl), ethidium bromide, then visualized using a gel documentation system. The study outline and screening methods for diagnosing suspects (MTB) of outdoor comorbid suspects is shown in Supplementary Figure 1.

Statistical analysis

Descriptive statistics was used to evaluate demographic characteristics. Mean and standard deviation for continuous data was measured. Number (n) and percentage (%) was used for categorical data. Sensitivity and specificity of sputum AFB smear, culture and GeneXpert MTB/RIF and PCR assay was calculated, and confidence interval (CI-95%) was also determined. The collected data were carefully analyzed using SPSS (version 25, Chicago Inc., IL, USA) to determine the prevalence of most essential variables like age, sex, clinical signs and symptoms, and demographic characteristics of PTB in the outdoor comorbid patients. *P* values (< 0.05) was considered significant.

RESULTS

Demography of the comorbid suspects and its association with M. tuberculosis

Among the comorbid suspects (n = 700), one hundred and thirty-nine (19.85 %) suspects were between 16 years and 30 years ($P > 0.05$), (30%) were between 31 years and

Table I. PCR primers for target genes.

Targeted genes	Primers sequences (5′-3′)	Product size (bp)	Annealing temperature (°C)	References
IS 6110	F: CCIGCGAGCGTAGGCGTCGG R: CTCGTCCAGCGCCGCTTCGG	123	68	Nyasulu *et al*. (2015)
rpo B	F: CAGACGTTGATCAACATCCG R: TACGGCGTTTCGATGAAC	305	58	MacLean *et al*. (2020)

45 years ($P > 0.05$), while 260 (37.71%) were around 60 years. Further, 6% of the suspects were between 61 years to 75 years ($P = 0.064$)(Table II).

Table II. Age and gender-wise distribution of all suspected comorbid isolates.

Age of suspects (year)	Male (n)	(%)	Female (n)	(%)	Total (%)
1-15	12	1.71	14	2.00	3.71
16-30	44	6.28	95	13.57	19.85
31-45	80	11.43	131	18.57	30.00
46-60	98	14.00	166	23.71	37.71
61-75	16	2.29	26	3.71	6.00
>75	8	1.14	6	0.86	2.00
Total suspects (700)	260	37.14	440	62.86	100.00

In the present study, among the suspects, gender wise, females were more effected (59%) ($P = 0.001$), than male (41%) and married couples were among the majority of suspects. Further, illiteracy rate was very high (44%). Nutrition (healthy food), economic conditions and occupation are the key factor that are interdependent and nutritious food could help the body to fight against the disease. Nevertheless, in the study participants, these factors were very poorly observed. Living conditions among the majority of suspects was poor and overcrowded. Additionally, majority of the suspects had familial history with TB (Table III).

Statistical association among the studied demographic characteristics showed a varied significant correlation. However, the most significant and positive association was observed among the variables including marital type, smoking habits with TB (OR, 1.59; 95% CI, 0.35-1.00), (OR, 0.40; 95% CI, 0.35-0.47) ($P < 0.05$), respectively. In summary, detailed prevalence and statistical correlation among the demographic variables was observed in comorbid isolates (Table III).

Association with comorbidities and clinico-pathological features with progression of pulmonary tuberculosis

In this study, medical comorbidities like asthma, diabetes (DM), hypertension, hepatitis and so on, were observed among the comorbid isolates, 37%, 28.9%, 21%, 11%. All the comorbid features were statistical analyzed and varied statistical correlation ($P > 0.05$) was noted. However, statistically significant association was recognized in a variable, asthma and night sweat OR, 0.587, (95% CI, 0.476- 0.724), ($P < 0.001$) (OR, 1.93; 95% CI, 1.173-1.179), ($P = 0.030$) (Table III).

Table III. Characteristic of study participants with clinical signs and symptoms.

Characteristics	Number n n (%)	Confidence level (95.0%) Low→High	*P*-value
Gender			
Male	287 (41)	1.439 (1.163 - 1.779)	< 0.05
Female	413 (59)		
Marital status			
Married	392 (56)	1.273 (1.069-1.515)	= 0.001
Single	308 (44)		
Comorbid status			
Asthma	259 (37)	0.587 (0.476-0.724)	< 0.001
Diabetes mellitus	196 (28)	0.389 (0.308-0.491)	
Hypertension	147 (21)	0.026 (0.206-0.343)	
Hepatitis	77 (11)	0.0124 (0.091-0.168)	
Kidney problem	12 (1.7)	0.017 (0.009-0.034)	
Others	9 (1.3)	0.013 (0.006-0.028)	
Education level			
No education	348 (49.72)	0.99 (0.85-1.15)	> 0.05
Primary	127 (18.14)		
Middle	118 (16.86)		
Matric	58 (8.29)		
Above matric	49 (7)		
Nutrition status			
Poor	406 (58)	1.38 (1.15-1.65)	< 0.001
Good	294 (42)		
Economic conditions			
Poor vs Good	322 (46)	2.19 (1.67-2.86)	=0.0001
Medium	231 (33)		
Medium vs Good	147 (21)	1.57 (1.18-2.09)	=0.002
Occupations			
Labor	155 (22.14)	0.38 (0.31-0.46)	< 0.05
Housewife/retired	301 (43)	0.31(0.14-0.16)	
No occupation	77 (11)	0.16 (0.13-0.21)	
Business	28 (4)	0.05 (0.04-0.08)	
Student	25 (3.57)	0.05 (0.03-0.07)	
Employee	51 (7.28)	0.10 (0.08-0.14)	
Others	63 (9)	0.13 (0.09-0.17)	
Smoking			
Smokers	272 (38.86)		
Non smoker	428 (61.14)		
Smoker vs non-smoker		0.40 (0.35-0.47)	< 0.001

Table continues on next page.....

Characteristic	Number n (%)	Confidence Level (95.0%) Low→High	*P*-value
Residence			
Rural	288 (41.14)		
Urban rural vs urban	412 (58.86)	0.49 (0.42-0.57)	0.000
Living/household conditions			
Normal	342 (48.86)		
Poor normal vs poor	358 (51.14)	1.10 (0.89-1.35)	> 0.05
Vaccination status (BCG)			
Yes	280 (40)		
No vaccinated vs non vaccinated	420 (60)	2.25 (1-82-2.79)	0.0001
Suspects familial history with TB			
Yes	252 (36)		
No	448 (64)	1.32 (0.95-1.42)	>0.05
Clinical signs and symptoms			
Chronic cough	315 (45)	0.037 (1.141-1.149)	>0.05
Fever	504 (72)	0.037 (1.138-1.146)	
Night sweat	378 (54)	1.93 (1.173 - 1.179	= 0.030
Weight loss	355 (55)	0.028 (1.148-1.204)	
Chest pain	406 (58)	0.037 (1.502-1.576)	
Weakness	532 (76)	0.067 (1.322-1.458)	
Anorexia	434 (62)	0.034 (1.276-1.346)	

In addition, weakness and fever was noted as the most common clinical sign (76% and 72%, respectively) followed by anorexia, chest pain, weight loss, night sweat, and chronic cough (62%, 58%, 55%, 54% and 45% respectively), among the comorbid isolates (Table III).

Traditional diagnosing assays in pulmonary tuberculosis

The results of sputum samples of all pulmonary TB suspects for AFB smear, MTB culture (LJ) and GeneXpert MTB/RIF assay are shown in Table IV. Eighty-nine (12.71%) sputum samples were found to have AFB smear positive results and remaining suspects sputum samples were AFB smear negative on ZN staining. Thus, the prevalence of PTB in suspects using direct AFB smear microscopy was 12.71% (per 100,000) (Table IV).

All the diagnosed positive and negative sputum AFB smear samples, using ZN smear microscopy were confirmed by its inoculation (direct method) on solid concentrated medium (LJ). Identification of the TB culture growth was conformed from the colony morphology, growth rate, and acid-fast staining for presence of TB bacilli (Kent, 1985; Khan *et al*., 2020). One handed and twenty-two sputum samples on solid media (LJ medium) MTB culture positive growth in the form of specific *M. tuberculosis* (MTB) colonies and positive growth rate was 17.43% (Table IV).

GeneXpert MTB/RIF assay for diagnosing pulmonary TB

Of the total 122 MTB positive sputum samples were processed through GeneXpert MTB/RIF assay. Of them 116/122 (95.08%) were detected positive for MTB by GeneXpert MTB/RIF assay in sputum samples. However, 03 samples of 122 sputum samples were MTB/RIF positive (2.45%) results and two sputum culture positive samples showed negative results. Further, one sputum culture positive gives no result/ error result, using Xpert assay. Therefore, the sensitivity of Xpert MTB/RIF assay was 95.08% (Table IV). Validation of positive MTB cases by GeneXpert MTB/RIF assay is worrying sign of disease in comorbid isolates. Further, identification of MTB/RIF positive cases is more alarming issue and serious precautionary and proper treatment approaches are required to inhibit its spread and management in the healthy individuals.

Table IV. Frequency distribution, sensitivity, specificity and comparison of pulmonary MTB cases by AFB smears, L.J culture and GeneXpert and PCR assay.

Pulmonary tuberculosis **diagnosing approaches**	**Positive result n (%)**	**Negative result n (%)**	**Specific performances**		**Culture contamination/ GeneXpert, PCR, error, n (%)**
			Sensitivity	**Specificity (%)**	
Z.N smear staining	89 (12.71)	611	12.71	87.29	-
LJ culture	122 (17.43)	532	17.43	82.57	46 (6.57)
GeneXpert MTB/RIF					
GeneXpert MTB	116 (95.08)	2	95.08	4.92	
GeneXpert MTB/RIF	03/122	119	2.45	97.55	1
PCR	121 (99.18)	1	99.18	0.82	
PCR RIF (*rpoB* gene)	3/3 (100)		100	-	

Overall, diagnostic performances of traditional assays of the sputum AFB smear, MTB culture (LJ) and an advanced molecular GeneXpert MTB/RIF assay for active pulmonary *M. tuberculosis* shown that sensitivity of GeneXpert MTB/RIF assay was higher (Table IV). Likewise, it gives more accurate and prompt results. However, it should consider that using sputum samples for GeneXpert MTB/RIF assay must be free of any food particles and other materials that may interfere the results of GeneXpert MTB/RIF assay.

Amplification of target genes in comorbid isolates and evaluation its sensitivity to GeneXpert MTB/RIF assay

All MTB positive sputum samples including the geneXpert MTB positive samples 116/122 (95.08%) as well as geneXpert MTB/RIF positive and negative samples (03) etc. were processed for PCR. Interestingly, in qPCR assays all geneXpert MTB/RIF samples (GeneXpert MTB samples, MTB/RIF samples plus error/ no result sputum sample) (121/122) were detected by PCR and sensitivity was (99.18%) and (100%) (Table IV).

Normally, GeneXpert MTB/RIF assay showed 119/122 (95.08%) sensitivity. However, qPCR/ PCR RIF assay sensitivity was 99.18% and 100%. Although GeneXpert MTB/RIF assay is an advanced, fully automated and real-time PCR-based assay. Nevertheless, in this study, it detected negative result/errors even though samples were MTB culture positive (processed in duplicate). Still, it detected the results in a short time. Therefore, to get accurate results in this assay, the sputum samples must be sterile from food contaminants and other non-innate objects.

DISCUSSION

Tuberculosis is recognized as disease of poverty and more common in developing world due to limited diagnosing resources and poor health care facilities. There are many challenges of timely diagnosis of tuberculosis, its drug resistance especially in smear negative clinical samples using conventional techniques because they are less sensitive and time consuming (Uddin *et al*., 2013; Schön *et al*., 2017). The current study has shown a considerable prevalence of active PTB cases among the comorbid suspects with TB.

Pakistan is among the top five countries with higher burden of TB globally and larger incidence and prevalence of this disease in the motherland has been endorsed due to numerous factors such as poverty, lack of awareness, social stigma and poor healthcare set-up (Ahmad *et al*., 2018; Grobusch and Kapata, 2018; Niamatullah *et al*., 2018; Khan *et al*., 2020). Our results were in accordance with above studies. Efforts are required to give awareness, retaining of the proper health, can minimize the spread of tuberculosis and other infectious diseases among the peoples.

TB suspects who are non-compliance about the disease and specially those who are diagnose with disease but do not complete the proper treatment, are alarming challenge for the country. Similarly, Balochistan having border with Afghanistan (war infected) and Iran, a larger number of immigrants have taken up ad hoc dwelling in Quetta, Pakistan. The unfettered movement of the immigrants across the borders and our province, potentially disrupt the course of TB treatment; that can significantly increase the disease spread and moreover into more complicated form (MDR) (Getnet *et al*., 2017; Niamatullah *et al*., 2018; Shafee *et al*., 2019).

In the current study, higher numbers of PTB suspects were females (59%) than male (41%) (Table II). Similar results were shown by former studies (Dogar *et al*., 2012; Shafee *et al*., 2014; Ahmad *et al*., 2016), which shows the increased rate of PTB in female. These results are in line with our previous studies (Khan *et al*., 2020). Which might be due to poverty, lack of awareness, neglected their disease, pregnancy, poor access to health facilities and negligence (Chang and Cataldo, 2014). Many clinical comorbidities have been reported in patients suspected for pulmonary tuberculosis in this study. Asthma (37%) was the most common comorbidity followed by diabetes mellitus (28%), hypertension (21%), hepatitis (11%). Whereas, 36% patients were with other disease such as kidney diseases etc. The presence of diabetes has also been shown as contributory risk factor for tuberculosis in many studies (N.R. *et al*., 2017; Al-Salihi and Mankhi, 2020). Similarly, studies showed that kidney disease was also considered as one of the comorbidities of tuberculosis (Romanowski *et al*., 2016; Bhattacharya *et al*., 2020). This shows that comorbidities are one the major risk for developing TB.

Awareness about the spread of infectious disease is more necessary. However, in our study majority of the suspects were noted with poor literacy rate, poor nutrition, remote hilly and rural areas and life style, these variables are parallel to previously studies (Oliva *et al*., 2008; Chang and Cataldo, 2014).

In the current study, around 37.7% of suspects of TB were 45-60 years years old. In which 23.71% were females. These results were in line to previous studies (Shafee *et al*., 2014; Ahmed *et al*., 2017). The high ratio might be due to their poor immune status. Use of nutritious food and improving the life style can alleviate the immune system.

In the present study, PTB related clinical signs and

symptoms (cough, chest pain, fever and so on) were prominent in majority of the study participants. Several studies have illustrated the same risk factors (Mushtaq *et al*., 2011; Rizvi *et al*., 2015). Monitoring these clinical signs and symptoms at initial stages could reduce the incidence of tuberculosis.

The most widespread comorbidities that interrelate with tuberculosis include; diabetes mellitus, HIV infection, depression, anxiety, malnutrition, smoking, alcohol abuse, and so on. The increasing evidence showed that comorbidities are an external concern that, when incorporated in tuberculosis management, make it more complicated (Van Rensburg *et al*., 2020; Cáceres *et al*., 2022). Comorbidities like diabetes, depression, increases the danger of treatment failure of tuberculosis, the emergence of multidrug-resistant tuberculosis, and even death in people with the disease (Sarker *et al*., 2016; Cáceres *et al*., 2022). Our results were parallel to these studies, where medical comorbidities like asthma, diabetes, hepatitis were most commonly observed. In addition, among the comorbid isolates, TB related clinical signs and symptoms (cough, chest pain, fever and so on) were also notes in majority of the isolates. Previous studies have illustrated the same risk factors (Mushtaq *et al*., 2011; Rizvi *et al*., 2015). Monitoring these comorbidities along with TB-related clinical signs and symptoms at initial stages could reduce the incidence and development of tuberculosis.

In the present study, we used gold standard procedures like ZN staining and LJ media techniques for identification of tubercle bacilli in suspects sputum samples (Kent, 1985; Ssengooba *et al*., 2012; Dheda *et al*., 2017). The prevalence of pulmonary TB sputum positive smear (PTB-SS+ve) infections on ZN staining was 12.71%, and remaining sputum samples were AFB negative per 100,000 of the population. These smear-positive cases are highly threatening that can easily transmit the disease among the healthy people, therefore its immediate diagnosis and proper treatment is extremely required to decrease the tuberculosis burden globally (Connell *et al*., 2011). Therefore, it is suggested to properly diagnose the sputum negative smear samples on advance molecular tool, like GeneXpert MTB/RIF, which is highly sensitive to detect the TB bacilli in small amount as well as RIF resistance, if present in suspected sputum sample. Fortunately, the MTB prevalence was lower than projected in the study, where it was 34.53 per 100,000 populations (Saleem *et al*., 2013; Shafee *et al*., 2014; Niamatullah *et al*., 2018). The current study results showed higher incidence of TB infections among the suspects in outdoor comorbid patients in Quetta. This rise in prevalence might be due to malnutrition, poor lifestyle and lack awareness among the study participants about the transmission of TB in comorbid isolates. Proper awareness among the peoples is highly necessary about the spread of infectious diseases like TB, which can reduce its incidence.

Studies have shown higher number of sputum negative smear samples on gold standard culture technique give positive MTB results, which shows that sputum culture assay is more sensitive and specific (Cheesbrough, 2006; Nyasulu *et al*., 2015). In the current study, the growth rate of *M. tuberculosis* bacilli was 17.43% on LJ culture medium (Table III). These results were similar to those shown in previous studies (Akhtar *et al*., 2007; Khan *et al*., 2017). Although, culture method is gold standard technique for diagnosis of MTB in active PTB, its use is hampered by the long time (Organization, 2015; Khan *et al*., 2020). Overall, outcomes of current study show higher dominance of MTB infections in outdoor TB suspects using ZN staining technique, whereas MTB LJ growth technique was highly sensitive in identification of TB. However, contamination rate was greater. Therefore, it is necessary to control the contamination, for this well biosafety laboratories are required.

In this study, an advance molecular Xpert MTB/RIF assay was used to process the LJ culture positive sputum samples, in which (95.08%) culture positive samples showed positive MTB results on Xpert MTB/RIF assay, this was more supportive and sensitive for identification of MTB in sputum samples. These results were consistent with previous studies (Reechaipichitkul *et al*., 2017). Likewise, in another descriptive study MTB/RIF assay was recognized as more sensitive and fast procedure for the early diagnosis of TB (Soeroto *et al*., 2019). This molecular approach might the best possible alternative with high sensitivity and specificity as compare to sputum smear microscopy and slow growth MTB culture technique. This was the first study in this area, which was focused on patients with many comorbidities. Overall, GeneXpert MTB/RIF is highly efficient and more sensitive assay in TB burden countries. Furthermore, to get accurate results from this assay, the sample must be sterile from food contaminants and other non-innate objects. In future, more advanced and novel tools are necessary to develop, that could detect multiple drug resistant TB (MDR-TB) and even extensively drug resistance TB (XDR-TB) in suspected sputum/ body fluids (for extra pulmonary TB) and blood serum of TB suspects. That will open new avenue for treatment of pulmonary and extra pulmonary TB infections.

In this study, the quantitative polymerase chain reaction assay, targeting the *1s6110* gene and *rpo*B gene, showed the highest sensitivity (99.18%). Similarly, the higher PCR sensitivity has been reported in previous studies

(Sah *et al*., 2017; Pérez-Osorio *et al*., 2012). Overall, this is the first study in this area, where this novel GeneXpert MTB/RIF assay was use in TB comorbid isolates and its sensitivity was confirmed through conventional polymerase chain reaction (PCR). It is highly efficient and more sensitive assay in TB burden countries. Further, it is designed for analysis of sputum samples only. In addition, to get accurate results in Xpert assay, the sputum sample must be sterile from food contaminants and other non-innate objects.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study reflects high prevalence of pulmonary *M. tuberculosis* among the comorbid isolates, which is very alarming and needed an urgent, effective intervention and more proper diagnosis and treatment is immediately required to monitor the transmission of this disease. Comorbidities, such as asthma, diabetes, and hepatitis were identified in the majority of the isolates. Similarly, among clinical signs and symptoms of tuberculosis, fever, anorexia, chest pain, and weight loss were distinguished. GeneXpert MTB/RIF assay was more advance and reliable and sensitive in diagnosis of pulmonary tuberculosis and RIF resistance in comorbid isolates than the other standard conventional assays. Nevertheless, qPCR assay sensitivity was more than Xpert assay. This is this first study in the study area, which was carried out in comorbid patients. Further, research is needed throughout the province to use GeneXpert MTB/RIF assay for determining the prevalence of MTB and MDR and XDR TB in diversified individuals of Balochistan, Pakistan.

DECLARATIONS

Acknowledgement

The authors extend their heartfelt gratitude to the Head of Pathology Department Dr. Muhammad Ashraf, Fatima Jinnah General and chest hospital, Quetta, Provincial Reference Laboratory (PRL), Baluchistan, its staff, and National TB Control Program (NTP), who generously shared their expertise, resources and provided valuable support and technical guidance that enabled the successful completion of this study. We also extend our sincere gratitude for the continuous technical assistance from the Department of Microbiology, Faculty of Life Sciences, BUITEMS, and the School of Life Sciences, Lanzhou University, China. This research was made possible by the dedication and commitment of all individuals involved.

Financial support

This research work did not obtain any financial support.

IRB approval

This study received Institutional Review Board (IRB) approval from the Department of Pathology, Fatima Jinnah General and Chest Hospital, Quetta, Balochistan, Pakistan.

Ethical considerations

This study was approved by the Ethical Review Committee of the Department of Pathology, Fatima Jinnah General and Chest Hospital, Quetta, Balochistan, Pakistan.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted tool was used in the writing, analysis, or preparation of this manuscript.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20250703171631

Statement of conflict of interest

The authors have declared no conflict of interest.

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Pakistan J. Zool., pp 1-12, 2026. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20250703171631

Supplementary Material

A Study on the Prevalence and Molecular Confirmation of *Pulmonary tuberculosis* Using GeneXpert MTB/RIF and its Comparison with Polymerase Chain Reaction and Conventional Assays Among the Outdoor Comorbid Isolates from Quetta, Pakistan

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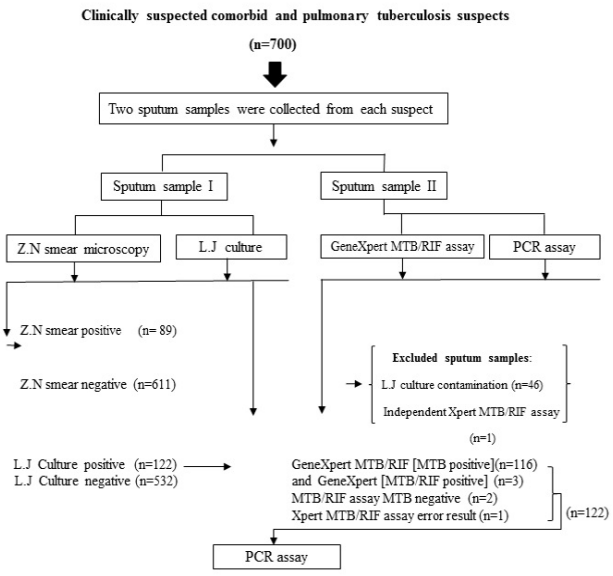

Supplementary Fig. 1. Flow diagram showing screening methods in comorbid suspects (MTB) of outdoor patients.

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0030-9923/2026/0001-0001 $ 9.00/0