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Evaluation of diagnostic efficacy of GeneXpert MTB/RIF assay with Ziehl Neelsen staining & microscopy in pulmonary tuberculosis

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Abstract
Tuberculosis is a bacterial highly chronic prevalent infectious disease caused by an intracellular aerobic bacterium known as mycobacterium tuberculosis. Because of this characteristic it prefers tissues which are always in contact with high oxygen levels as in lungs. Conventional methods for TB diagnosis such as microscopy have low sensitivity. Though culture is considered as gold standard, it is time consuming. Newer molecular diagnostic methods such as GeneXpert assay are rapid and highly sensitive and are now playing a pivotal role in TB diagnosis. The rapid detection of Mycobacteria in clinical specimens is essential for the early diagnosis and treatment of patients. Hence based on above data the present study was planned for Evaluation of diagnostic efficacy of GeneXpert MTB/RIF assay with Ziehl Neelsen Staining & Microscopy in Diagnosis of Pulmonary Tuberculosis.
The present study was planned in Department of Microbiology, Aiims, Patna, Bihar, India. The study was conducted from September 2016 to august 2017. In the present study 660 suspected cases of the Pulmonary tuberculosis were included and evaluated.
The data generated from the present study concludes that GeneXpert MTB/RIF assay is an effective tool for diagnosis of tuberculosis and has better sensitivity than smear and microscopic examination. It is very much useful to detect more cases of smear negative TB. It can also detect rifampicin resistance in TB bacilli (Multi-drug resistant tuberculosis).

Keywords: tuberculosis, Z-N staining, M. tuberculosis, GeneXpert assay, pulmonary TB, etc

Introduction
Tuberculosis, a multisystemic disease with myriad presentations and manifestations, is the most common cause of infectious disease–related mortality worldwide. Although TB rates are decreasing in the United States, the disease is becoming more common in many parts of the world. In addition, the prevalence of drug-resistant TB is increasing worldwide.
The World Health Organization (WHO) has estimated that 2 billion people have latent TB and that globally, in 2009, the disease killed 1.7 million people [1, 2]. Coinfection with the human immunodeficiency virus (HIV) has been an important factor in the emergence and spread of resistance [3].
Mycobacterium tuberculosis, a tubercle bacillus, is the causative agent of TB. It belongs to a group of closely related organisms—including M africanum, M bovis, and M microti —in the M tuberculosis complex. The lungs are the most common site for the development of TB; 85% of patients with TB present with pulmonary complaints. Extrapulmonary TB can occur as part of a primary or late, generalized infection.
The primary screening method for TB infection (active or latent) is the Mantoux tuberculin skin test with purified protein derivative (PPD). An in vitro blood test based on interferon-gamma release assay (IGRA) with antigens specific for M tuberculosis can also be used to screen for latent TB infection. Patients suspected of having TB should submit sputum for acid-fast bacilli (AFB) smear and culture. The usual treatment regimen for TB cases from fully susceptible M tuberculosis isolates consists of 6 months of multidrug therapy. Empiric treatment starts with a 4-drug regimen of isoniazid, rifampin, pyrazinamide, and either ethambutol or streptomycin; this therapy is subsequently adjusted according to susceptibility testing results and toxicity. Pregnant women, children, HIV-infected patients, and patients infected with drug-resistant strains require different regimens. Laws vary from state to state, but communicable-disease laws typically empower public health officials to investigate suspected cases of TB, including potential contacts of persons with TB. In addition, patients may be incarcerated for noncompliance with therapy. New TB treatments are being developed [4], and new TB vaccines are under investigation.
TB is an ancient disease. Signs of skeletal TB (Pott disease) have been found in remains from Europe from Neolithic times (8000 BCE), ancient Egypt (1000 BCE), and the pre-Columbian New World. TB was recognized as a contagious disease by the time of Hippocrates (400 BCE), when it was termed "phthisis" (Greek from phthinein, to waste away). In English, pulmonary TB was long known by the term “consumption.” German physician Robert Koch discovered and isolated M tuberculosis in 1882.
The worldwide incidence of TB increased with population density and urban development, so that by the Industrial Revolution in Europe (1750), it was responsible for more than 25% of adult deaths. In the early 20th century, TB was the leading cause of death in the United States; during this period, however, the incidence of TB began to decline because of various factors, including the use of basic

infection-control practices (eg, isolation).

The US Centers for Disease Control and Prevention (CDC) has been recording detailed epidemiologic information on TB since 1953. Beginning in 1985, a resurgence of TB was noted. The increase was observed primarily in ethnic minorities and especially in persons infected with HIV. TB control programs were revamped and strengthened across the United States, and rates again began to fall.

As an AIDS (acquired immunodeficiency syndrome)–related opportunistic infection, TB is associated with HIV infections, with dual infections being frequently noted. Globally, coinfection with HIV is highest in South Africa, India, and Nigeria. Persons with AIDS are 20-40 times more likely than immunocompetent persons to develop active TB [5]. Correspondingly, TB is the leading cause of mortality among persons infected with HIV [6].

Worldwide, TB is most common in Africa, the West Pacific, and Eastern Europe. These regions are plagued with factors that contribute to the spread of TB, including the presence of limited resources, HIV infection, and multidrug-resistant (MDR) TB.

MDR-TB is defined as resistance to isoniazid and rifampin, which are the 2 most effective first-line drugs for TB. In 2006, an international survey found that 20% of M tuberculosis isolates were MDR. [16] A rare type of MDR-TB, called extensively drug-resistant TB (XDR-TB), is resistant to isoniazid, rifampin, any fluoroquinolone, and at least one of 3 injectable second-line drugs (ie, amikacin, kanamycin, or capreomycin). [1] XDR-TB resistant to all anti-TB drugs tested has been reported in Italy, Iran, and India [7].

Multiple factors contribute to the drug resistance of M tuberculosis, including incomplete and inadequate treatment or adherence to treatment, logistical issues, virulence of the organism, multidrug transporters, host genetic factors, and HIV infection. A study from South Africa found high genotypic diversity and geographic distribution of XDR-TB isolates, suggesting that acquisition of resistance, rather than transmission, accounts for between 63% and 75% of XDR-TB cases [8].

In a 2008 report by the WHO, the proportion of TB cases in which the patient was resistant to at least 1 antituberculosis drug varied widely among different regions of the world, ranging from 0% to over 50%; the proportion of MDR-TB cases ranged from 0% to over 20%. The WHO calculated that the global population-weighted proportion of MDR-TB was 2.9% in new TB cases, 15.3% in previously treated patients, and 5.3% in all TB cases [9].

In the United States, the percentage of MDR-TB cases has increased slowly, from 0.9% of the total number of reported TB cases in 2008 to 1.3% of cases in 2011. Although the percentage of US-born patients with primary MDR-TB has remained below 1% since 1997, the proportion of cases in which the patient was foreign born increased from 25.3% in 1993 to 82.7% in 2011 [10].

XDR-TB is becoming increasingly significant [9]. According to the US National TB Surveillance System (NTSS), between 1993 and 2006 a total of 49 cases (3% of evaluable MDR-TB cases) met the revised case definition for XDR-TB. The largest number of XDR-TB cases was found in New York City and California.

The cure rate in persons with MDR-TB is 50-60%, compared with 95-97% for persons with drug-susceptible TB [6]. The estimated cure rate for XDR-TB is 30-50% [1]. In people who are also infected with HIV, MDR-TB and XDR-TB often produce fulminant and fatal disease; time from TB exposure to death averages 2-7 months. In addition, these cases are highly infectious, with conversion rates of as much as 50% in exposed health-care workers.

As previously stated, multidrug resistance has been driven by poor compliance with TB therapies, resulting in difficulties in controlling the disease. Consequently, a threat of global pandemic occurred in the late 1980s and early 1990s. Reacting to these signals, the WHO developed a plan to try to identify 70% of the world's cases of TB and to completely treat at least 85% of these cases by the year 2000.

Out of these goals were born major TB surveillance programs and the concept of directly observed therapy (DOT), which requires a third party to witness compliance with pharmacotherapy. With worldwide efforts, global detection of smear-positive cases rose from 11% (1991) to 45% (2003), with 71-89% of those cases undergoing complete treatment.

Despite the importance of early isolation of patients with active TB, a standardized triage protocol with acceptable sensitivities has yet to be developed [11]. Moran *et al*. demonstrated that among patients with active TB in the emergency department (ED), TB was often unsuspected, and isolation measures were not used [12]. The difficulty in establishing such a protocol only highlights the importance of the emergency physician’s role in the prompt identification and isolation of active TB.

A large percentage of ED patients are at increased risk for having active TB, including homeless/shelter-dwelling patients, travelers from endemic areas, immunocompromised patients, health-care workers, and incarcerated patients. Therefore, emergency physicians must consider the management and treatment of TB as a critical public health measure in the prevention of a new epidemic [13].

For high-risk cases, prehospital workers can assist in identifying household contacts who may also be infected or who may be at high risk of becoming infected. Prehospital workers should be aware that any case of active TB in a young child indicates disease in 1 or more adults with close contact, usually within the same household. TB in a child is a sentinel event indicating recent transmission.

Infection with M tuberculosis results most commonly through exposure of the lungs or mucous membranes to infected aerosols. Droplets in these aerosols are 1-5 μm in diameter; in a person with active pulmonary TB, a single cough can generate 3000 infective droplets, with as few as 10 bacilli needed to initiate infection.

When inhaled, droplet nuclei are deposited within the terminal airspaces of the lung. The organisms grow for 2-12 weeks, until they reach 1000-10,000 in number, which is sufficient to elicit a cellular immune response that can be detected by a reaction to the tuberculin skin test.

Mycobacteria are highly antigenic, and they promote a vigorous, nonspecific immune response. Their antigenicity is due to multiple cell wall constituents, including glycoproteins, phospholipids, and wax D, which activate Langerhans cells, lymphocytes, and polymorphonuclear leukocytes

When a person is infected with M tuberculosis, the infection can take 1 of a variety of paths, most of which do not lead to actual TB. The infection may be cleared by the host immune

system or suppressed into an inactive form called latent tuberculosis infection (LTBI), with resistant hosts controlling mycobacterial growth at distant foci before the development of active disease. Patients with LTBI cannot spread TB.

The lungs are the most common site for the development of TB; 85% of patients with TB present with pulmonary complaints. Extrapulmonary TB can occur as part of a primary or late, generalized infection. An extrapulmonary location may also serve as a reactivation site; extrapulmonary reactivation may coexist with pulmonary reactivation.

Tuberculosis is a bacterial highly chronic prevalent infectious disease caused by an intracellular aerobic bacterium known as *Mycobacterium tuberculosis.* Because of this characteristic it prefers tissues which are always in contact with high oxygen levels as in lung. The most important tool for the control of tuberculosis is the microbiological diagnosis which is consists of conventional methods (acid-fast microscopy, culture, biochemical identification, anti-tuberculosis drug-susceptibility test) and modern molecular techniques. The rapid detection of Mycobacteria in clinical specimens is essential for the early diagnosis and treatment of patients. Hence based on above data the present study was planned for Evaluation of diagnostic efficacy of GeneXpert MTB/RIF assay with Ziehl Neelsen Staining & Microscopy in Diagnosis of Pulmonary Tuberculosis.

Methodology

The present study was planned in Department of Microbiology, AIIMS, Patna, Bihar, India. The study was conducted from September 2016 to August 2017. In the present study 660 cases of the Pulmonary tuberculosis were included and evaluated.

Morning sputum samples were collected from these patients after giving proper instructions so as to obtain good quality sputum in a Falcon tube of 50 ml capacity. At least 5 ml of sputum sample is collected. The falcon tube is labelled with patient's name, registration number and other identification details and sent to tuberculosis laboratory in the department of Microbiology.

From each sputum sample, a direct smear is prepared on a clean, grease free glass slide using a clean disposable wooden applicator stick. The slide was air dried and heat fixed by passing it through Bunsen's burner flame 3 – 4 times. The slide is then placed horizontally on a staining rack and stained by Ziehl – Neelsen (Z-N) method as per the Revised National Tuberculosis Control Program (RNTCP) guidelines. [14] Acid fast bacilli are seen as bright red or pink rods against blue background.

The same sputum samples are then tested for detection of Mycobacterium tuberculosis DNA using GeneXpert MTB/RIF assay. This method is more sensitive in detecting presence of TB bacilli in sputum samples than just smear and microscopic examination. It can detect both Mycobacterium tuberculosis DNA and genetic mutations associated with Rifampicin resistance simultaneously in a short span of just two hours using sputum samples [15]. Though conventional culture-based drug sensitivity testing is considered as a gold standard investigation to detect MDR, the sensitivity and specificity of GeneXpert MTB/RIF assay is comparable with conventional methods [16].

GeneXpert MTB/RIF assay is a fully automated cartridge based molecular diagnostic test for TB. In this assay, about 3 – 5 ml of sputum sample is mixed with twice the volume of sample reagent. It is shaken vigorously and incubated at room temperature for 10 minutes. After 10 minutes it is again shaken vigorously and incubated for another 5 minutes. 2 ml of this processed sample is then added to GeneXpert cartridge which is then loaded in the device. The results are finally interpreted by the GeneXpert system based on fluorescent signals which are displayed on the system monitor after about 90 minutes [16].

Following was the inclusion and exclusion criteria for the present study.

Inclusion Criteria: Patients with Pulmonary tuberculosis with symptoms such as cough with or without expectoration for >2 weeks, weight loss, fatigue, hemoptysis and loss of appetite.

Exclusion Criteria: Patients having any other complications.

Results & Discussion

In this study, a total of 660 sputum samples were tested by smear and microscopic examination with Z-N staining as well as by GeneXpert MTB/RIF assay. Out of these total samples, 185 (28%) samples were tested positive for acid fast bacilli (AFB) by smear and microscopic examination after Z-N staining. 475 (72%) sputum samples were negative for AFB. Table 1 shows the result s of smear examination with Z-N staining in case of total 660 sputum samples.

Table 1: Results of Smear examination with Z-N staining

Observation	No. of Cases
Positive	185 (28%)
Negative	475 (72%)
Total	660

When the same sputum samples were subjected to GeneXpert assay, in 228 (34.55%) sputum samples, *M.tuberculosis* DNA was detected whereas 432 (65.45%) sputum samples gave negative result in GeneXpert. Table 2 shows the result of GeneXpert testing in case of total samples.

Table 2: Result of GeneXpert MTB/RIF test

Observation	No. of Cases
M. tuberculosis detected	228 (34.55%)
M. tuberculosis not detected	432 (65.45%)
Total	660

In case of 228 sputum samples which were positive in GeneXpert assay, only 175 samples showed presence of acid-fast bacilli in Z-N staining. 53 sputum samples were positive by GeneXpert but negative in smear examination. Whereas in case of 10 sputum samples, smear examination was positive and GeneXpert test was negative. The combined results of both sputum smear examination and GeneXpert testing are shown in Table3.

Table 3: Combined Result of GeneXpert testing & Sputum smear Examination

Observation	GeneXpert Positive	GeneXpert Negative	Total
Smear Positive	175	10	185
Smear Negative	53	422	475
Total	228	432	660

Conventional methods such as smear microscopy for detection of M. tuberculosis in clinical specimens have low sensitivity. Molecular techniques, including the Cepheid GeneXpert system, have changed the field of TB with rapid diagnosis combined with high sensitivity and specificity results. In December 2010, the WHO endorsed the GeneXpert MTB/RIF assay for the rapid diagnosis of TB and MDR-TB. Now- a- days the GeneXpert facility is available for TB diagnosis at many Government Hospitals at District and even at Taluka places under Revised National Tuberculosis Control Program (RNTCP) and is provided free of cost to all patients. It has now come up as a major revolution in the field of TB diagnosis.

Microbiologic confirmation of tuberculosis is challenging because of low and variable sensitivity of culture and the difficulty in obtaining appropriate samples. In addition, turnover time of culture is usually in weeks. Xpert MTB/RIF (Mycobacterium tuberculosis/Rifampicin) is a point-of care diagnostic test that can be performed with minimal training and results are available within two hours [18]. Utility of Xpert MTB/RIF assay on respiratory specimens in adults is well established [19]. After endorsement by the World Health Organization [20], several studies have evaluated the utility of Xpert MTB/RIF assay for the diagnosis of pediatric tuberculosis [21]. However, data on diagnostic utility of Xpert MTB/RIF assay using bronchoalveolar lavage (BAL) fluid in children are limited [22].

GeneXpert is fully automated molecular test for tuberculosis case detection and drug resistance testing, developed through collaboration in a public–private partnership. Xpert MTB/ RIF, an automated molecular test for Mycobacterium tuberculosis (MTB) and resistance to rifampin (RIF). Uses heminested real-time polymerasechain- reaction (PCR) assay to amplify an MTBspecific sequence of the rpoB gene. Testing is carried out on the MTB/RIF test platform (GeneXpert, Cepheid), which integrates sample processing and PCR in a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The MTB/RIF cartridge is then inserted into the GeneXpert device, which provides results within 2 hours.

In our study, out of the total 660 sputum samples from patients of suspected pulmonary tuberculosis, smear examination (Z-N staining) was positive in 185 (28%) patients. But when GeneXpert assay was performed on same sputum samples, it was positive for 228 (34.55%) cases. Thus, GeneXpert was found to be superior for detection of *M. tuberculosis* in sputum samples as compared to microscopy and smear examination alone. Many other such studies show similar findings.

Doris Hillemann in 2011 compared Gene Xpert MTB/RIF (Xpert) assay system with conventional liquid and solid culture methods. 521 specimens (91 urine, 30 gastric aspirate, 245 tissue, 113 pleural fluid, 19 cerebro-spinal fluid and 23 stool specimens) were submitted. The combined sensitivity and specificity of the Xpert assay were calculated to be 77.3% and 98.2%, respectively [23].

Raquel Moure *et al*, in 2012 conducted a study; in this research, out of 108 smear-negative extra pulmonary samples 63 (58.3%) were positive with the Xpert MTB/RIF assay (Gene Xpert) for Mycobacterium tuberculosis [24].

In a similar study by Vadwai in 2011, the sensitivity of the Xpert assay was 81% (228/283 specimens), 64% for smear-negative cases and 96% for smear-positive cases), with a specificity of 99.6% [25].

Gu Y *et al*, in 2015 conducted a study for bone and joint tuberculosis, in which they found sensitivity of smear to be very less (26%) as compared to Xpert test (86%) [26].

The Gene Xpert MTB/RIF assay marks an important development in the field of rapid molecular TB diagnostics. This assay was rapidly endorsed by the WHO (World Health Organization) in December 2010 as a replacement for sputum smear microscopy, particularly in settings with high rates of HIV-associated TB and multidrug-resistant TB developed for testing sputum samples [27].

This multifunctional diagnostic platform is an automated, closed system that performs real-time PCR and can be used by operators with minimal technical expertise, enabling diagnosis of TB and simultaneous assessment of rifampicin resistance to be completed within 2 hours [28, 30]. The test detects DNA specific for Mycobacterium tuberculosis by polymerase chain reaction [32].

More recently, however, evaluations of the assay have extended to a variety of non-respiratory clinical samples from patients with extra-pulmonary tuberculosis. A definitive diagnosis can be made by detection of M. tuberculosis in extra-pulmonary samples [29].

Acid-fast stains are non-specific and the reader cannot determine the species of Mycobacteria present in a positive smear. Other study by Ibrahim (2012) [33] in Sudan showed 2.4%, regarding the current study it was obviously increase the number of mycobacterium other than tuberculosis and these may be attributed to HIV status, which the atypical is more dominant among HIV patients and other immunocompromised patients. Study by Maghzob 2010) who reported 84% of patients with HIV were found infected with MTB while 16% were co-infected with NTM [34]. TB treatments take too long to cure, are too complicated to administer, and can be toxic. Many people have negative interactions between commonly used antiretroviral and TB treatment. People with TB must take drugs from 6 months to 2 years or longer—or risk developing more difficult to treat drug-resistant TB, treatment for drug-resistant TB can take up to two years, and is so complex, expensive, and toxic that many patients are unable to access treatment. Further, the cost of curing MDR-TB can be staggering — literally thousands of times as expensive as that of regular treatment in some regions — posing a significant challenge to health systems.

If TB is to be eliminated as a global problem, earlier diagnosis and treatment will be essential. Tuberculosis can be diagnosed by Smear microscopy either by Ziehl-Neelsen's or Fluorescent staining; conventional culture either liquid or solid culture methods or molecular methods like GeneXpert, PCR and CBNAAT. Even though smear microscopy is easy and economical, it has some problems in detecting tuberculosis in patients with low bacterial load and differentiation is also difficult with other mycobacteria.

The most common way for diagnosing TB worldwide is through sputum smear microscopy using the fluorescence microscope (Auramine) or the Ziehl-Neelsen method. However, this method is susceptible to human error and other factors beyond control that can result in false negatives. The impact of false negative in TB diagnosis can have far reaching consequences and is very detrimental to the global initiative as it may mean further spread of TB infections from untreated cases.

Conclusion

GeneXpert MTB/RIF assay is an effective tool for diagnosis of tuberculosis and has better sensitivity than smear and microscopic examination. It is very much useful to detect more cases of smear negative TB. It can also detect rifampicin resistance in TB bacilli (Multi-drug resistant tuberculosis). Early detection and appropriate treatment of drug sensitive as well as MDR-TB is an important part of TB control activities and it will also help to control the spread of this highly communicable disease in the community.

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