Review Article

The Value of Using Tagman Real-time PCR, Phenol-Auramine Stain and Ziehl-Neelsen Stain in The Diagnosis of Pulmonary Tuberculosis in Fine-Needle Aspiration Material

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ABSTRACT

Pulmonary tuberculosis represents a growing health problem among Iraqi population with the emergence of an increasing number of new cases each year. The diagnosis of tuberculosis relies upon the identification of TB bacilli in the sputum of suspected patients, traditionally done by Ziehl-Neelsen (ZN) stain, along with other diagnostic modalities including culture, PCR study and phenol auramine (PA) stain, nevertheless; some patients may present with advanced disease (post primary, lung mass or cavitary lesions with mediastinal lymphadenopathy), sometimes mimicking lung tumours, necessitating fine needle aspiration (FNA) to exclude other pathologies. In order to evaluate the usefulness of different real-time PCR, PA and ZN stains, 102 patients subjected to fine needle aspiration were included in this study. Tagman real-time PCR, PA stain and ZN stain were applied to 23 out of 102, in which malignancy was excluded and the diagnosis of tuberculosis was suspicious. Tagman real-time PCR was highly accurate, specific and sensitive (100%), while both PA and ZN stain revealed sensitivity, specificity and accuracy of 54.5% vs. 45.4%, 35.9% vs. 27.77%, and 78.2% vs. 73.9% respectively.

Keywords: Tagman real time PCR

INTRODUCTION

TB is an infectious disease caused by the bacillus Mycobacterium tuberculosis. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). The disease is spread in the air when people with pulmonary TB expel bacteria, for example by coughing. Overall, a relatively small proportion of people infected with M. tuberculosis will develop TB disease. However, the probability of developing TB is much higher among people infected with HIV. TB is also more common among men than women, and affects mainly adults in the most economically productive age groups. The most common method for diagnosing TB worldwide is sputum smear microscopy (developed more than 100 years ago), in which bacteria are observed in sputum samples examined under a microscope. Following recent breakthroughs in TB diagnostics, the use of rapid molecular tests to diagnose TB and drug-resistant TB is increasing. In countries with more developed laboratory capacity, cases of TB are also diagnosed via culture methods (the current reference standard). Without treatment, TB mortality rates are high. In studies of the natural history of the disease among sputum smear-positive/HIV-negative cases of pulmonary TB, around 70% died within 10 years; among culture-positive (but smear-negative) cases, 20% died within 10 years[10].

Since ancient times, there have been references to TB or illnesses resembling TB from several parts of the world from many civilizations. The earliest references to TB can be found in the language *Samskritam* (*Sanskrit*). In the ancient Indian scriptures, *The Vedas*, TB was referred to as *Yakshma* (meaning wasting disease). Description of a TB-like disease has been documented in ancient Chinese and Arabic literatures[1-3]. In English literature, the word "consumption" (derived from the Latin word *consumer*) has also been used to describe TB. The word "tuberculosis" appears to have been derived from the Latin word *tubercula* (meaning "a small lump")[4,5].

Fracastorius (1443-1553) believed that TB was contagious. Thomas Willis (1621-1675) had documented the clinical presentation of consumption in detail in his treatise *Pthisiologica*. Richard Morton (1637-1698) had described several pathological appearances of TB[2,4,6]. John Jacob Manget gave the description of classical miliary TB in 1700[7]. In 1720, Benjamin Marten conjectured that TB could be caused by "certain species of animalcula or wonderfully minute living creatures". In 1865, Jean Antoine Villemin presented his results suggesting that TB was a contagious disease[1,2]. However, it was Robert Koch who announced the discovery of the tubercle bacillus during the monthly evening meeting of the Berlin Physiological Society on 24th March 1882[8,27]. On this day, after thousands of years, *Mycobacterium tuberculosis*, the

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organism causing TB finally revealed itself to humans. Commemorating the centenary of this event, since 1982, 24th March is being celebrated as "World TB Day" world over. Wilhelm Conrad Roentgen's discovery of X-rays, facilitated radiographic visualization of changes caused by TB in a living person. Thus, it was in the early years of 20th century that basic concepts related to ætiological agent of TB, consequent pathological changes in humans and detection of the organism became established.

Epidemiology

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360,000 of whom were HIV-positive. TB is slowly declining each year and it is estimated that 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment. However, given that most deaths from TB are preventable, the death toll from the disease is still unacceptably high and efforts to combat it must be accelerated if 2015 global targets, set within the context of the Millennium Development Goals (MDGs), are to be met.

In Iraq, tuberculosis is still representing a major health problem that has its burden on the health care system. According to the WHO annual report in 2013, it is estimated that the incidence of tuberculosis is 45 per 100,000 population with a total of notified cases of 8554 in 2013, although decreasing along the last ten years; this figure is still high compared with endemic areas. Due to the rapid growth of population the prevalence of tuberculosis among Iraqi population is seemingly on a slow steady rise estimated to be around 75 per 100,000 population, with an estimated total cases of tuberculosis to be 25,000, ranging between 12,000-44,000[9]. One study conducted in Babylon governorate illustrated that there was 60 cases of newly diagnosed cases of pulmonary and extrapulmonary tuberculosis during the period extending from Feb. to Aug. 2012[26].

Globally, about one-third of the world's population has latent TB. Symptoms of tuberculosis appear in only about 10% of infected individuals. However, persons with compromised immune systems, such as people living with HIV, malnutrition or diabetes, or people who use tobacco, have a much higher risk of developing symptoms. The annual report of the WHO on TB in 2012 has stated that there were 8.8 million (range, 8.5-9.2 million) incident cases of TB, at an estimated median incidence of 125/100,000 population. Of these, 13% were among people living with HIV. The proportion of TB cases co-infected with HIV was highest in countries in the African Region which had accounted for 82% of TB cases among people living with HIV. Women accounted for an estimated to 3.2 million incident cases[11].

Natural History

After exposure to the infective microorganism, only about 30% of exposed persons develop infection, of which only about 10% develop symptoms of primary disease, leaving 90% cases of potentially infective carrier latent TB (LTBI). Diagnosis and treatment of LTBI is important in the eradication of TB. Reactivation of the disease in LTBI will result in the development of post-primary TB. Reactivation in healthy adults occurs in about 10% of cases, while it occurs in more than 30% of HIV infected individuals. Post-primary tuberculosis may take the form of pulmonary TB or extra-pulmonary or disseminated TB (EPTB).

Diagnosis

Latent TB infection:

Tuberculin skin test (TST).

Interferon-gamma release assays (IGRAs).

Bacille-Calmette-Guerin (BCG).

Active TB infection:

Smear staining with Ziehl-Neelsen stain.

Fluorescence microscopy.

Culture on Lowenstein-Jensen media; the gold standard method.

Nucleic acid amplification tests (NAAT):

Line probe assays (LiPA).

GenExpert/Cepheid®.

Isothermal NAAT (under validation).

Genotyping methodologies.

Serodiagnostic tests: Not recommended by the WHO.

Imaging studies.

Extra-pulmonary TB

Detection of AFB by Ziehl-Neelsen stain and fluorescent microscopy.

Lowenstein-Jensen culture.

Histopathology and cytology, including FNA and examination of body fluid.

Adenosine-deaminase level in body fluids.

Interferon-γ in body fluids.

Imaging techniques.

Ziehl-Neelsen Stain and Phenol-Auramine Stain

The Ziehl–Neelsen stain, also known as the acid-fast stain, was first described by two German doctors: the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854–1898). It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria, responsible for tuberculosis. The principle of staining procedure depends on the presence of mycolic acid in bacterial cell wall of mycobacterium species, which gives the cell wall the thick waxy structure that prevents most hydrophilic stains like Gram's stain. The presence of phenol in carbol fuchsin with the aid of heating assists the integration of basic fuchsin within mycolic acid, then decolorized with acid alcohol and counter stained with methylene blue or malachite green[12,13,14]. The presence of mycolic acids gives M. tuberculosis many characteristics that defy medical treatment. They lend the organism increased resistance to chemical damage and dehydration, and prevent the effective activity of hydrophobic antibiotics. In addition, the mycolic acids allow the bacterium to grow readily inside macrophages, effectively hiding it from the host's immune system. Mycolate biosynthesis is crucial for survival and pathogenesis of M. tuberculosis, and could be targeted by many anti-mycobacterial agents[15,16].

The same principle is applicable for phenol-auramine staining of mycobacterium species. The fluorescent stain, auramine, is incorporated within mycolic acid in the

presence of phenol, which is not decolorized with acid alcohol and detected under fluorescent microscope[17,18].

The Role of Fine-Needle Aspiration

Tissue samples for cytologic examination obtained by fine-needle aspiration could be indicated in some cases of pulmonary tuberculosis whenever the diagnosis of tuberculosis cannot be established easily using the conventional laboratory methods and imaging techniques. Furthermore; imaging techniques could be more confusing in some of the cases with consolidating masses or associated with cavitary lesions and mediastinal lymphadenopathy. Exclusion of malignancy by cytologic examination is essential and could be very important in the diagnosis of pulmonary tuberculosis. Traditionally, any cytologic material obtained from a lung mass, which reveals a constellation of chronic inflammatory cells and histiocytes it is mandatory to be stained with Ziehl-Neelsen stain to illustrate the presence or absence of AFB.

FNA cytology from extra-pulmonary TB has been very effective in the diagnosis of tuberculosis and tuberculous granulomas[19,20,21], while FNA of pulmonary lesions is still of limited value, unless done by endoscopic ultrasound guided procedure[22,23,24].

Molecular Diagnosis

Different methods have been invented to target specific mycobacterial genes for the accurate and rapid detection of tuberculosis. The nucleic acid amplification based TB diagnostic tests (NAAT) are based on the amplification of short specific sequences of DNA or RNA of M. tuberculosis complex by PCR and the amplified products are then detected by agarose/acrylamide gel electrophoresis, or by various hybridization methods. Several in-house PCR assays and commercial kits have been used for rapid diagnosis of TB.

Integrated automated NAAT, the GeneXpert (Cepheid Inc., Sunnyvale, CA, USA) platform combines automated sample preparation, real-time PCR amplification, identification of M. tuberculosis and detection of rifampicin resistance in less than 120 minutes[25]. GeneXpert has the advantage of being simple to use even in field conditions and appears promising technology for rapid diagnosis of TB[28].

Objectives:

Evaluate the usefulness of tagman real-time PCR in the diagnosis of pulmonary tuberculosis in FNA material.

Evaluate the sensitivity of ZN and PA stains in identifying TB bacilli in FNA material of lung lesions.

Characterize and localize TB bacilli in FNA material using ZN and PA stains.

Methodology

One hundred and two cases were included in this study, of which malignancy was excluded by cytologic examination in 23 cases. Twelve cases were having a questionable diagnosis of tuberculosis by fine needle aspiration, but finally failed to achieve the criteria for final diagnosis of tuberculosis and eleven patients were finally diagnosed with pulmonary tuberculosis by different laboratory methods and imaging techniques. Smears of FNA material were retrieved and stained both by ZN stain and PA stain according to the method recommended by the manufacturer. Unstained and unfixed smears were treated with saline solution to dissolve the attached material, DNA extraction was done and tagman PCR for M. tuberculosis accomplished using a kit designed for PCR detection of MTB by Bioneer® corporation, South Korea.

RESULTS

Out of 102, 23 cases were finally refined according to the fine needle aspiration cytologic material obtained by the procedure, having non-malignant lesions, of which eleven cases were finally diagnosed having pulmonary tuberculosis by different laboratory and imaging methods. All eleven cases gave a positive result for M. tuberculosis by tagman real-time PCR technique (100 % sensitivity, specificity and accuracy). Six patients were positive for PA (54.5% sensitivity, 35.9% specificity and accuracy of 78.2%), and five patients showed a positive result using ZN stain (45.4% sensitivity, 27.77% specificity and accuracy of 73.9%). The positive predictive value of all the above mentioned tests (tagman real-time PCR, PA stain and ZN stain) was 100%.

Out of 23 cases refined by FNA, the cytologic examination was highly suggestive of tuberculosis in 13 cases, with a false positive rate of 15.3% and a sensitivity of 100% , specificity of 75% and a positive predictive value of 84.6%.

In all six PA-positive cases, mycobacteria were detected intracellularly within histiocytes and extracellularly in necrotic material, while the only case which was positive for ZN stain; the bacilli were seen within the necrotic material aspirated.

DISCUSSION

The introduction of nucleic acid amplification techniques in the diagnosis of tuberculosis has greatly contributed to the rapid and accurate diagnosis of pulmonary and extrapulmonary tuberculosis, eliminating the waiting time in cultivating TB bacilli, although the latter is regarded a reference method. Amplification of certain segments of nucleic acid of TB bacilli genomic material can either be detected by gel electrophoresis to identify the amplified target segment or by real-time fluorescence detection after PCR amplification each cycle. Two major techniques are available in real-time PCR: CYBR green which binds in a non-specific manner to the newly formed amplicons emitting fluorescence, and tagman real-time PCR, in which there is a specific probe designed to anneal specifically to the amplified product releasing the fluorescent dye to be detected each cycle. The latter real-time technique is more specific than CYBR green and offers specific detection of target genes. Non-specific polymerase chain reaction amplification can be detected easily by CYBR green technique and can be recognized as non-specific after studying the melting curve of the product, while tagman technique offers more specific detection. Most companies started to offer tagman real-time PCR kits for rapid and accurate detection of TB, along with the processing software.

CONCLUSIONS

Tagman Real-time PCR specifically designed to detect M. tuberculosis is more sensitive and is regarded as the golden method for the diagnosis of pulmonary tuberculosis in FNA material. The use of PA stain is more sensitive than ZN stain in illustrating the presence of mycobacteria in smears of FNA material of lung lesions, and is regarded as a useful tool to confirm the diagnosis of TB.

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