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Diagnosis of *Tuberculosis* meningitis by conventional methods and Real-time PCR in Iraqi patients

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Abstract

This study was carried out to diagnosis the *Tuberculosis* meningitis from immune competent and immune compromised patients at different age groups. Sixty samples were collected and enrolled in this study over five months from January to May 2013, conventional methods included cell count and differentiation, biochemical analysis, staining and culturing of the samples and molecular methods included real –time PCR, after DNA extraction from CSF using a freeze–thaw technique to lyse cells, and the Wizard genomic DNA purification kit to purify DNA. Real-time PCR which target the IS6110 in *Mycobacterium tuberculosis* reveals that 25(41.66 %) patients sample out of 60 samples were real –time PCR positive, regarding diagnosis by staining and culture 12(20%) samples from the total CSF samples (n=60) were detected by Ziehle-Neelsen stain (ZN) and culturing. In conclusion Tuberculosis meningitis should be included as one of the most cases in chronic meningitis in Iraq and real-time PCR assay could be great help in early diagnosis of Tuberculosis meningitis even when stain and culture was negative.

Keywords: Insertion sequence 6110(IS6110), Real time polymerase chain reaction (RT PCR), Tuberculosis meningitis (TBM), *mycobacterium tuberculosis complex* (MTBC).

Introduction

Mycobacterium tuberculosis (MTUB) is a Bacterial pathogen that causes most cases of tuberculosis this pathogen most commonly attacks the lungs, and then leads to granuloma formation and finally calcified lesions. Spread of the bacteria within a year of initial infection results in "primary disease". However, the organisms may remain dormant but viable for decades. If the immunity of the patients compromised, the bacteria may escape into the lungs causing "re-activated pulmonary tuberculosis" (Wilkinson *et al*., 2000; Bentrup and Russel, 2001). In some cases the bacteria spread to other host tissues through the lymphatic system and blood, resulting in "miliary or extra-pulmonary tuberculosis (EPTB)" (Thwaites, 2000). *Tuberculosis* meningitis (TBM) one of the severe forms of extra-pulmonary tuberculosis and it is most harmful infectious disease involving central nervous system with very high morbidity and mortality as well as high frequency of neurologic sequelae the disease is a serious cause of death in developing countries mortality from TBM is 30% and greater in individuals with immunosuppressive status (Razin *et al*., 2011). The development of TBM is a two- step process. *M. tuberculosis* enters the host by droplet inhalation, the initial point of infection being the alveolar macrophages. Localized infection escalates within the lungs, with dissemination to the regional lymph nodes to produce a primary complex. In persons who develop TBM, bacilli seed to the meninges or brain parenchyma resulting in the formation of small or subendymal foci of metastatic caseous lesions (Van de Beek *et al*., 2006).The major challenge in the diagnosis of extra pulmonary tuberculosis (EPTB)is the frequently atypical clinical presentation simulating other inflammatory and neoplastic conditions, which frequently results in a delay or deprivation of treatment (Thwaites *et al*., 2002). Delays in diagnosis and treatment are regarded as major contributing factors to the high mortality reported in many recent series (Brancusi *et al*., 2012).

Despite new diagnostic techniques and treatment regimens, mortality and morbidity associated with chronic meningitis remains high. As the number of *Mycobacteria*in cerebrospinal fluid is extremely low staining methods is therefore difficult and not sufficiently sensitive, the current gold standard for the diagnosis of these

organisms remains the isolation of it from cerebral spinal fluid (CSF) by culture (Hoşoğlu *et al*., 2003). However, there is an urgent need for more rapid diagnostic techniques as culture can take up to 8 weeks (Ananth *et al*., 2009).

Materials and Methods

Patients and clinical specimen: Cerebrospinal fluid (C.S.F) from sixty patients who admitted to city hospital, Al-Yarmouk teaching hospital, Al-Emamain Al-Kadhymain Teaching Hospital, Al-Elwia pediatrics hospital and Ibn Al–Khateeb hospital Baghdad were enrolled in this study, the levels of glucose and proteins as well as cell cytology were used to roll out patients with other types of meningitis. Questionnaire for each patients including name, age, gender, location, clinical signs and symptoms, BCG vaccination status, was filled for each patients. Five to twelve milliliters of CSF samples were collected in sterile containers.

Processing of Sample: Sample processing occur according to standard operating procedures (SOPs) which was included macroscopic appearance, volume, protein, sugar cell count and differentiation gram stain as well as Ziehl-Neelsen stain and culturing of the sample in special media (Prince, 2010). CSF separated in three tubes the first one underwent chemical analysis (sugar and protein), cell count and differentiations; the second one was centrifuged for 10min. at 2000-3000 rpm, sediment was resuspended for direct diagnosis by Ziehl-Neelsen stain (ZNS) and indirect by inoculated one to two drops of the sediments in Lowenstein Jensen incubated at 37°Cfor 6-8 weeks and the slopes were examined weekly, while the third tube was stored in– 20ºCtill DNA extractions. All samples were inoculated and processed in class II biological safety cabinets (BSC) under sterile conditions.

DNA was extracted from each sample using a freeze–thaw technique (Reischl *et al*., 1994) to lyse cells, and the Wizard genomic DNA purification kit (Promega) to purify DNA. The concentration and purity of the purified DNA was quantified by the use of nanodrop instrument following the instruction of the manufacturer, the primers sequence (5'to3') usedtheIS6110 PCR was selected for amplifications (IS1 FR 5'-CCTGCGAGCGTAGGCGTCGG3', IS2 RE5'CTCGTCCAGCGCCGCTTCGG3'). The insertion sequence elements IS6110 present in the genomes of all members of the *M. tuberculosis complex* and most studies have used IS6110 as a target for PCR based diagnosis of MTBC (Kusum *et al*., 2012).

RT-PCR using TaqMan as say was made according to manufactured instructions (Anatolia gene works-Turkey), for one reaction as follow PCR Mix (20µl), Detection mix 1(1.6µl) Detection mix 2(1.2µl) Internal control (0.2µl) with 18µl of DNA (sample, positive or negative control) total volume (41µl) Tubes were closed well and spin. The final PCR conditions were initial denaturation of double stranded DNA molecules at 95°C for 14:30 min. this was followed by cycling, which included denaturation at 97°C for 30 min, annealing and synthesis (data collection) at 53 °C for 2 min. After the 50th cycle, a terminal hold step was then performed at 22°C for 5minutes.Befor starting a real time PCR reaction choose FAM filter for unknown sample and CY5 for standard. The standard curve was constructed using a 10-fold dilution by plotting the log of starting quantity of template against the CT value obtained during amplification of each dilution. Reaction efficiencies of standards were 93% for *Mycobacterium Tuberculosis*. Figure (1) the real-time PCR was performed using Agilent Real-time PCR (Techne-UK).

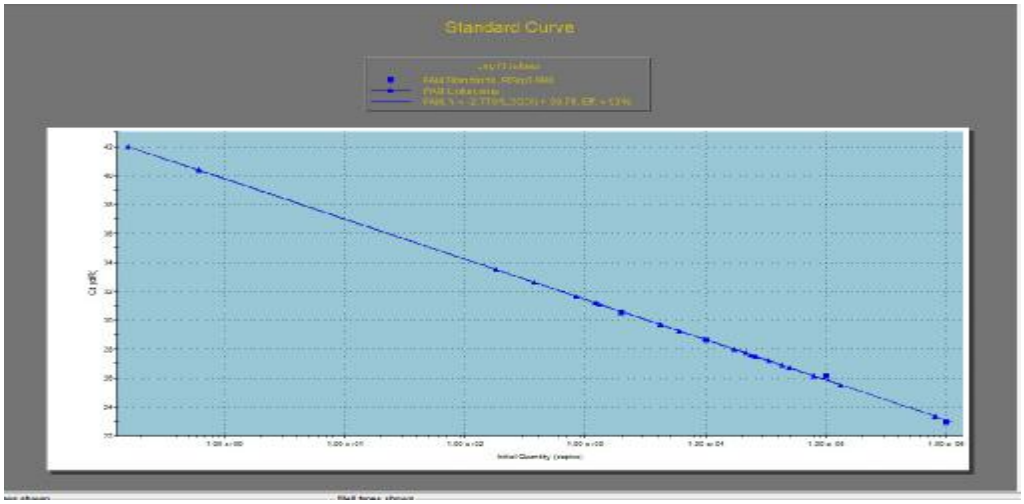

Figure (1): Standard curve explains efficiency of real time PCR.

Results and Discussion

Demographic information of the patients: The total number of patients with presumptive diagnosis of *Tuberculosis* meningitis was sixty patients were enrolled in this study, The principle findings were the age which ranged between (63) and (67) years with mean of (35) years, three patients (4.5%) were under the age of ten years. From the whole study populations 20 were females (33.33%) and 40 were males (66.66%), there was a statistical significant difference between age and sex distribution, ratio of males to females 2.2:1. Most cases were from low socioeconomic status region from Baghdad Figure (2).

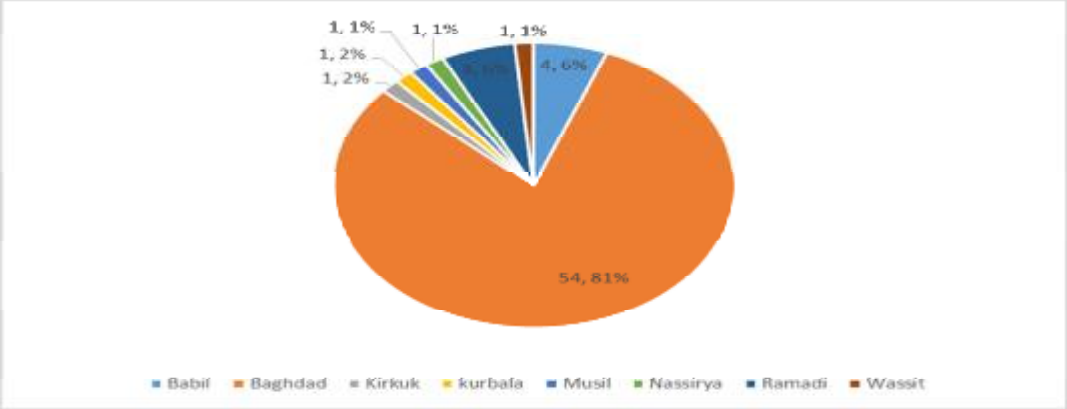

Figure (2): Distribution of Tuberculosis in Iraq province.

Fifty (83.33%) cases of those enrolled within this study were received BCG vaccine improved by BCG scar, only 10 of the remainders (16.66%) have no history for such vaccine. concerning patients with *Tuberculosis* meningitis, eleven (44%) have been no BCG vaccine and the other14 (56%) were received this vaccine.

Cell count and differentiation were done on all CSF samples (n=60), the mean of white blood cells was (319.70) and predominantly, lymphocytic pleocytosis with a mean of (71.5) versus (26.53 for neutrophil) the mean volume of CSF was (5-12ml), glucose level was (48.58) and mean of protein level was (123.28). Analysis of the subgroup of patients that were subsequently proved to be real- time PCR positive for *Tuberculosis* meningitis (n=25) displayed typical changes in CSF parameters with elevated white blood cell predominantly lymphocytes as well as high level of protein with low level of sugar. Smear and culture Results: Twelve samples (20%) out of 60 CSF samples were detected by Ziehle-Neelsen stain (ZN) as bright red, straight or slightly curved and by culture on Lowenstein Jensen (LJ) media which were as colorless, rough surface colonies after incubation period of 14-28 days. Sensitivity and specificity of Ziehle –Neelsen stain compared to culture (gold standard) were 75 % and 94.55% respectively. Negative predictive value (NPV) was 94.55 % while positive predictive value (PPV) of 75 %.

Real-time PCR: IS6110 primer was used for the detections of *Mycobacterium Tuberculosis Complex*, results improved that 25(41.66%) samples out of 60 were real –time PCR positive. All culture positive samples (n= 12) were positive by real- time PCR. Clinical observations demonstrated that the majority of the patients that showed positive results for real-time PCR were poorly responded to the first line anti-TB therapies which were improved in seven samples by Gene Xpert MTB/RIF Assay (a test applied in reference lab/ center of Tuberculosis &chest disease in Baghdad) as a rifampicin resistant for those patients. The sensitivity of real-time PCR was 100% and the specificity was 76.36%.The negative predictive value (NPV) was 100 % with a positive predictive value (PPV) of 48 %.

*Mycobacterium tuberculosis*one of the most common causes of chronic meningitis and is the most common form of central nervous system (CNS) infections encountered worldwide and is a potentially fatal form of CNS infections, with serious long-term consequences. The fast and accurate laboratory diagnosis of the cause of chronic meningitis is of the utmost importance in order to direct therapy (Kashyap *et al*., 2004).

In current study the mean age of the patients from whom the specimens were analyzed was (35) years with range of (63-67) years, it was found that among 25patients positive *tuberculosis* meningitis by real-time PCR 15(60%) was male and 10(40%) female. This shows inclination of *Tuberculosis* meningitis towards male, similar finding was

observed by Tripathy *et al*. (1985); Al-Alusi and Belasim (1997). In current study the included cases were from the low socioeconomic status regions. Which may associated with malnutrition. There is a clear evidence that there was a close link between chronic meningitis and nutrition, it's clear that individuals who are malnourished are more susceptible to these type of infection (Tarakad *et al*., 2013).

Forty eight (80%) out of 60 patients of those enrolled within this study were received BCG vaccine, the remainders 12(20%) have no history for such vaccine. Concerning patients with Tuberculosis meningitis, which improved as 25 patients eleven (44%) have been no BCG vaccine and the other 14(56%) were received this vaccine, this result may shed light into the role of the BCG vaccine that this vaccine may give some protection against Tuberculosis meningitis and its come with agreement with previous studies done by Zodpey *et al.,* (1996); Zhang *et al.,* (2000).

In this study CSF profile was significantly abnormal in definite diagnosis and in all cases that have been studied. CSF picture revealed high level of protein and low level of sugar and white blood cell was high in number with predominance lymphocytic pleocytosis in all samples positive for Tuberculosis meningitis and these finding is agree with those in most previous studies by Rajagopalan (2001), Winston, *et al.* (2012). This indicates that each of cell count and chemical picture of CSF were offers a good diagnostic prediction in patients with Tuberculosis meningitis, and this comes in agreement with previous studies done by Berenguer *et al.* (1992) and Cherian and Thomas (2011). The sensitivity and specificity for ZN smears was 75% and 94.55%. Our ZN sensitivity and specificity compares well to a study performed by Caws, *et al*(2007) who found a sensitivity of 52.6% and 90% specificity. Out of 25 positive samples for Tuberculosis meningitis by real- time positive PCR only twelve (48%) were culture positive, the acceptable reason is may be due to paucibacilliary nature of TBM, and the required number for positive mycobacterial culture is at least 20micro-organisms/ml. Microbiological identification of *M. tuberculosis* in CSF is highly volume-dependent. (Thwaites *et al*., 2002).

IS6110 real-time PCR results improved that 25(41.66%) samples out of 60 were positive. The sensitivity of real-time PCR was 100% and the specificity was 76.36%. Results obtained in this study show agreement with some previous studies by Lin, *et al*. (1995) and it is in disagreement with various others such as those of Bonington *et al*. (1998); Chaidir, *et al*. (2012), and this might reflect the fact that some of cases enrolled in this study were not belong Tuberculosis meningitis.

In this study clinical observations demonstrated that the majority of the patients showed positive results for real-time PCR were poorly responded to the first line anti-TB therapies (rifampicin resistant) which were improved in seven samples by Gene Xpert MTB/RIF Assay. The explanations for this result, that *Mycobacterium tuberculosis* invades the CNS system are highly virulence and required more effective therapy (Caws *et al.*, 2008) in Iraq this resistance may be due to the antibiotic abuse or because the rifampicin was the drug of choice in brucellosis and other chronic disease. This result quite accord with studies done by Caws *et al.* (2007); Baker *et al*. (2004) who found that *Mycobacterium Tuberculosis* isolated from CNS infections show degree of resistant to the first line of anti-TB therapy.

Conclusions

Our study made it evident that *Tuberculosis* meningitis should be included as one of the most cases in chronic meningitis in Iraq and real-time PCR is a rapid and sensitive test for diagnosis of this disease.

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