Diagnosis of Cryptococcus meningitis by conventional methods and Real-time polymerase chain reaction

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Abstract— Cryptococcal infections of CNS are the most common form of fungal infections, mostly affects the immunodeficient individuals. Rarely, even normal hosts can become so diseased. Infection can also occur in patients on long-term steroid therapy, diabetes, cancer, renal failure, immunosuppressive treatment, and solid organ transplant patients. This study was carried out to diagnosis the Cryptococcus 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 as well as latex agglutination test for detecting Cryptococcal antigen in CSF and molecular methods included SYBR Green real –time PCR Four (6.66 %) patients sample out of 60 samples were real –time PCR and latex agglutination test positive, Regarding diagnosis by staining and culture two (3.33%) samples from the total CSF samples (n=60) were detected by nigrosin stain and culturing. In conclusion Cryptococcosis should be included as one of the most important causes in chronic meningitis in Iraq and real-time PCR assay could be great help in early diagnosis of Cryptococcus meningitis.

Index Terms— Cryptococcus neoformans, Cerebrospinal fluid, Real time polymerase chain reaction, Cryptococcus meningitis (CM), Latex agglutination test, standard operating procedures.

INTRODUCTION

Cryptococcus neoformans is encapsulated yeast that is present in the environment worldwide and can cause disease in both immunocompetent and immune compromised hosts (1). It was first described in 1894 when Busse and Buschk independently reported on the same case of 31-year-old woman with a history of enlarged lymphatic glands that had developed a large ulcer over her tibia (2). There three recognized varieties and four serotypes of C. neoformans serotypes based on the antigenicity of the capsular polysaccharides: C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D) and C. neoformans var. gattii (serotypes B and C) as well as a hybrid of C. neoformans var. grubii and C. neoformans var. neoformans serotype AD (3, 4). In the last years new proposed changes to the nomenclature suggest that C. neoformans should be divided into two distinct species including C. neoformans (serotypes A, D and AD) and C. gattii (serotypes B and C) (5,6).

Cryptococcal infections of CNS are the most common form of fungal infections, with the advent of the acquired immunodeficiency Cryptococcosis occurs worldwide and mostly affects the immunodeficient individuals. Rarely, even normal hosts can become so diseased (7). Infection can also occur in patients on long-term steroid therapy, diabetes, cancer, renal failure, immunologic diseases, immunosuppressive treatment, and solid organ transplant patients (8, 9). Early and rapid diagnosis is crucial for successful disease managements as the case fatality rate for untreated Cryptococcal meningitis is almost 100% and the delay in institution of appropriate treatment can lead to permanent neurological sequelae (10). Latex agglutination test (LAT) one of the most important serodiagnostic used in diagnosis of Cryptococcal infection was first described by Bloomfield et al. (11).

MATERAL AND METHODS

Cerebrospinal fluid (CSF) from sixty patients who admitted to City hospital, Al-Yarmouk teaching hospital, AL-Kadhimiya 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 was filled for each patients. Five to twelve milliliters of CSF samples were collected in sterile containers.

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 nigrosin stain, Cryptococcus latex test and culturing of the sample in special media (12). CSF separated in three tubes the first one underwent chemical analysis (sugar and protein), cell count and differentiations; the second one was centrifuged for 10 minutes. at 2000 -3000 rpm, the supernatant aspirated with a sterile pipette into a sterile tube, then was tested by Latex agglutinations assay (LAT) for Cryptococcal antigen, sediment was resuspended for direct diagnosis by negative stain (nigrosin stain) and indirect by inoculated one to two drops of the sediments in Sabouraud dextrose agar incubation temperature was 30 ˚C times for 2 days, while the third tube was stored in – 20ºC till 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 freezing–thawing technique (13) to cell lysis, 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, Two primers were designed according to internal transcribed spacer

regions of ribosomal DNA found in Cryptococcus neoformans were used Primer sequence (5′ to3′) CN-5 Forward (3′-GAAGGGCATGCCTGTTTGAGAG-5′) CN-6 Reverse (3′-TTTAAGGCGAGCCGACGTCCTT-5′) (BioCorp Canada) for the diagnosis of Cryptococcal meningitis (CM). KAPA-SYBR FAST qPCR master mix is a ready to use cocktail containing all components except primers and template. Assay was made according to manufactured instructions(KAPA BIOSYSTEMS, South Africa) for one reaction as follow Kapa Syper master mix (20µl), Forward primer(1.6µl) Reverse primer (1.2µl) Rox low (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 20Sec. this was followed by amplification steps for 40 cycle, which included 95°C for 30 Sec, 56°C for one Sec ,72 °C for 30 Sec. After completion of the amplification process, the reaction mixture was denatured at 95°C, held at 55°C for 30Sec, and then slowly heated to 95°C for melting curve constrictions. During this process fluorescence was continuously monitored and melting curves were constructed automatically then converted to melting peaks. Before starting a real time PCR reaction choose SYBR filter for unknown sample and ROX low 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 were 90% real-time PCR was performed using Agilent Real-time PCR (Techne-UK).

Discussion:

Cryptococcus 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 (8).

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 4 positive cases with Cryptococcus meningitis females were 3(75%) and only one male (25%) this finding agrees with those published by Mwaba et al(14) Othman et al (15). 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 (16). 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 Cryptococcus meningitis and these finding is agree with those in most previous studies by Rajagopalan (17), Winston (18). This indicates that each of cell count and chemical picture of CSF were offers a good diagnostic prediction in patients with Cryptococcus meningitis,

Results:

The total number of patients with presumptive diagnosis of Cryptococcus 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. the highest incidence was below ten years followed by the fourth decade, It was found that among 4 positive cases with Cryptococcus meningitis females were 3(75%) and only one male (25%) Most cases were from low socioeconomic status region from Baghdad. 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 Cryptococcus meningitis (n=4) 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.

Two (3.33%) samples out of 60 CSF samples were nigrosin stain positive which appeared as oval to spherical in shape surrounded by capsule also these samples were positive for C. neoformans which were creamy colored and mucoid colonies on Sabouraud agar incubation temperature was 30 ˚C times for 2 days. Concerning Cryptococcal Antigen Latex Agglutination test Four (6.66%) out of 60 samples were positive for latex agglutination test. Serial dilutions of the four positive specimens ranged from 1:4 to 1:32 respectively with a mean of 15 They were also positive for Cryptococcus neoformans by real- time PCR (5.97%) and the copy number ranged from 17-34 copy with a mean of 23.25 The highly titter samples latex test was showed highly copy number real-time PCR Table (4- 8). The sensitivity and specificity of latex agglutination test for Cryptococcal meningitis in current study compare with real-time PCR were 100 %. Negative and positive predictive values (NPV&PPV)) were 100 % for each.

Four patients (6.66%) were positive for Cryptococcal meningitis by each of real –time PCR assay and latex agglutination test out of 67 patients, only two samples (n=2) out of these four samples were positive in direct and indirect methods .Those patients with positive Cryptococcus meningitis were categorized in to two groups, first one included three patients with chronic debilitated disease (Diabetic, kidney transplant and systemic lupus erythematous) and the second group included one patient who was seven years old.

And this comes in agreement with previous studies done by Berenguer, et al (19) and Cherian (20).

Regarding diagnosis of Cryptococcus meningitis (CCM) by nigrosin staining method only two samples were positive which were confirmed later by Sabouraud dextrose agar culture. The yield rate by negative stain method (nigrosin stain) in comparison with real-time PCR was (50%) and this result was accordance with those in most previous studies by Sabetta and Adriole, (21) Dismukes,et al(22) who found that the yield rate of the negative stain was ranging from 40% to 79%. In this study Cryptococcus latex agglutination test was found to be the more sensitive in comparison with staining and culture methods, the titter results in positive samples Cryptococcus latex antigen was correlated with copy number of PCR method. These results agree with those reported by Saldanha, et al (7). in this study four out of 60 patients (6.66%) were positive for Cryptococcal meningitis by each of real –time PCR assay and latex agglutination test and only two samples (n=2) were positive by direct and culture methods. Many published paper improved that PCR methods is the powerful tool for detection of Cryptococcal neoformans in clinical samples because it allows the selective amplification of very small amounts of nucleic acids (23).In this study each of latex agglutination test and real time PCR had gave the same results, that improved the compatibility of these tests and this agree with the result reported by Saha, et al (24).

Three of those four patients who developed Cryptococcus were suffering from chronic debilitated diseases and the fourth was less than 10 years. These findings are consistent with several clinical studies which improved that resistance to Cryptococcosis depends primarily on cell-mediated immunity and Cryptococcus meningitis occur in patients with conditions that weaken this system, such as AIDS. Cryptococcal meningitis has also been reported in HIV negative patients in the background of organ transplant and chemotherapy related immunosuppression such as corticosteroid therapy and the ubiquitous nature of C. neoformans in the environment. Concerning the child under 10 years, the large number of pigeons in urban areas, increased likelihood of environmental exposure for children (25).

Conclusion:

Our study made it evident that Cryptococcal 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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