Wude Mihret , Guta Zenebe, Ashenafi Bekele , Markos Abebe , Liya Wassie, Lawrence K. Yamuah, Dawit WoldeMeskel, Yonas Kassahun, Girmaye Medhin, Howard Engers, Abraham Aseffa. *Ethiop Med J, Sup 1*

ORIGINAL ARTICLE

Chronic meningitis in immunocompromised adult Ethiopians visiting Tikur Anbessa Teaching Hospital and Ye'huleshet Clinic from 2003-2004

Wude Mihret (MSc)[1*], Guta Zenebe (MD)[2,3], Ashenafi Bekele (MD)[2], Markos Abebe (PhD)[1], Liya Wassie (PhD)[1], Lawrence K. Yamuah (PhD)[1], Dawit WoldeMeskel (MSc)[1], Yonas Kassahun (PhD)[1], Girmaye Medhin (PhD)[4], Howard Engers (PhD)[1], Abraham Aseffa (MD, PhD)[1]

ABSTRACT

Background: *Chronic meningitis is inflammation of the meninges where signs and symptoms develop and last for at least four weeks without alleviation. Little is known about the current etiology and incidence of the disease in adults living in developing countries.*

Objective: *The objective of this study was to elucidate the most common etiologies of chronic meningitis in adult Ethiopian patients and give an aid in the empiric therapy.*

Methodology: *A total of 53 adult patients (median age 32 years) having chronic meningitis and who were admitted at Tikur Anbessa Teaching Hospital and Ye'huleshet Clinic, Addis Ababa, Ethiopia were recruited between 2003 and 2004. Of the 53 patients, bacteriological, molecular and immunological investigations were done for 52 of the study participants to detect Cryptococcus neoformans, Mycobacterium tuberculosis, Toxoplasma gondii, Brucella and Neisseria meningitides infections.*

Results: *Forty eight of the participants were HIV positive and 15% (8/52) of the CSF were positive with Cryptococcal latex antigen detection test; in addition, M. tuberculosis DNA was detected using PCR from CSF of patients in few of the patients. Multiple infections were observed in study participants with < 0.1 to 1 CD4 to CD8 ratio.*

Conclusion: *Chronic meningitis mostly occurred in HIV infected patients, where most of the infections were attributed to Cryptococcus neoformans whereas M. tuberculosis appeared secondary.*

[1]Armauer Hansen Research Institute, P.O.Box: 1005, Jimma Road, Addis Ababa, Ethiopia

[2]Tikur Anbessa Teaching Hospital, Addis Ababa University, Medical Faculty, P.O.Box 1176, Addis Ababa, Ethiopia

[3]Ye'huleshet Clinic, Telephone address: +251-118-501337, P.O.Box 1337, Addis Ababa, Ethiopia

[4]Aklilu Lemma Institute of Pathobiology, Addis Ababa University, P.O.Box 1176 Addis Ababa, Ethiopia

* Corresponding author:

Wude Mihret

Armauer Hansen Research Institute, Jimma Road ALERT Campus, P.O.BOX 1005

Telephone +251-910-498641

Email address: wudebizumoges@gmail.com

INTRODUCTION

The disease meningitis is manifested in the form of acute (16), chronic and recurrent meningitis (15). Detection of the cases with these different forms of meningitis through clinical and laboratory based diagnosis would be of a valuable task in respect to patient management especially in immunocompromised individuals such as patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (14). Chronic meningitis is characterized by a progressive, sub-acute onset of leptomeningeal disease and persisting cerebrospinal fluid (CSF) abnormalities such as elevated protein level and pleocytosis for at least one month (1). Apart from using crude CSF and few clinical laboratory parameters, clinical diagnosis of chronic meningitis and its etiologic agent have remained obscure (1,2). Little is known about the current etiology of chronic meningitis in adults living in developing countries (17), where guidelines for patient management are lacking (16). Severe opportunistic and mixed infections often occur in immunocompromised patients infected with HIV (3, 4). As Ethiopia is one of the Sub-Saharan African countries, which is severely stricken with HIV/AIDS (5) and tuberculosis, we hypothesized that *Mycobacterium tuberculosis (M. tuberculosis)*, *Cryptococcus neoformans (C. neoformans)*, *Toxoplasma gondii (T. gondii)* and *Trepaneoma pallidum (T. pallidum)* are the most identifiable etiologic agents of chronic meningitis in this part of the world and we aimed to identify the common causative agents of chronic meningitis through bacteriologic, immunologic and molecular detection methods on CSF samples obtained from adult Ethiopians patients who are clinically diagnosed with chronic meningitis. The findings can thus be correlated to the specific,

frequent signs and symptoms in order to contribute to the validation of the current empiric therapy.

MATERIALS AND METHODS

Study participants

The study design was cross-sectional in which blood and CSF samples were collected from 53 individuals who were clinically diagnosed to have chronic meningitis and admitted at Tikur Anbessa Teaching Hospital and Ye'huleshet clinic from November 2003 to December 2004. The median age of the participants was 32 years (15-69 yrs), of whom 29 were females. Cerebrospinal fluids were collected from the patients as part of their routine diagnosis and upon the physician's recommendations and left over specimens were transported to AHRI/ALERT laboratory at room temperature within one hour after collection to be used in this study.

A tracer was assigned to further follow the patients, who were admitted during the study period after they were discharged from the hospital, relatively after a year since their enrollment into the study, for possible sequel of chronic meningitis in the subsequent year. This study received ethical approval from the AHRI/ALERT Ethics Review Committee and then the National Ethical Clearance Committee at the Ministry of Science and Technology Agency. All participants gave written informed consent before participating into the study.

Sampling and laboratory analyses

Of the 53 participants recruited into the study, 8 ml of CSF was collected from all cases and 20 ml of venous blood from 51 cases under sterile condition. However, laboratory analyses of CSF data were done for 52 cases due to inadequate sample for analyses. Samples from CSF

were aliquoted and frozen at -20ºC. An aliquot of sample was centrifuged at 13,000 rpm for 15 minutes for further bacteriological and mycological investigations, which were done on chocolate, blood and Thayer-martin agar and Sabouraud dextrose agar (SDA), respectively. Briefly, pellets from CSF specimens were inoculated into blood and chocolate agar and Thayer-martin medium incubated in 5% CO_2 at 37°C and in a dry incubator, respectively and bacterial growth was inspected after 24 hours, 48 and 72 hours. Gram staining was done for the CSF residue following routine procedures. On the other hand, a portion of CSF pellet inoculated into SDA media were incubated at 37°C dry incubator and inspected for possible growth of *C. neoformans* as described elsewhere (6). Routine india ink (nigrosin, Sigma) staining was also performed from CSF residue. In case of growth of fungal micro-organisms on SDA, sub-cultures were carried out in Niger seed agar and mycosil agar for confirmation. Further confirmation of the result was done using Latex-Crypto antigen detection test kit (Immuno-Mycologics) following the company's test instruction.

About three ml of the CSF samples were investigated for the growth of *M. tuberculosis*. Samples were centrifuged at 3000 rpm for 15 minutes at room temperature and routine acid fast bacilli smear was done from a portion of CSF residue and the remaining residue was re-suspended in supernatant, where 500 µl was inoculated into 7H9 broth and the remaining residue was transferred to Lowensen Jenson (LJ) and 7H10 agar based media, incubated at 37°C dry incubator. The growth of *M. tuberculosis* was inspected after 8 weeks.

Extraction of genomic DNA and PCR

A portion of CSF (~ 1.5 ml) that was aliquoted and frozen was thawed and DNA was extracted using phenol chlorophorm DNA extraction and ethanol precipitation method (11) and PCR was performed using IS6110 (an insertion sequence element used to identify members of the *M. tuberculosis* complex) (7). The two conventional sets of primers used were INS1/2 (5'-CGT GAG GGC ATC GAG GTG GC3'), INS2 (5'-GCG TAG GCG TCG GTG ACA. AA-3') and IS5/6 (5'-CGG AGA CGG TGC CTA AGT GG-3'), IS6 (5'-GAT GGA CCG CCA GGG CTT GC-3'), to amplify a 245 bp and 980 bp of the gene, respectively (8,9). Reactions were run in a total volume of 25 μL containing 100 ηg of purified DNA template and 2μL from each primer using a thermal cycler (Gene Amplification PCR system 9700, PE Bio. system, Norwalk, CT). Amplification of a housekeeping gene (β-globin) was used as internal control and to normalize total DNA copy numbers as a function of the total copy numbers of a nuclear gene. Primer pair used for β-globin gene amplification at 268-bp was GH20 (5'-GAA GAG CCA AGG ACA GGT AC-3') and PCO4 (5'-CAA CTT CAT CCA CGT TCA CC-3').

Analyses of plasma samples

Plasma samples were isolated by centrifugation at 1500 rpm for 5 minutes at room temperature, from which cerebrospinal fluid glucose, protein levels and plasma glucose levels were determined using photometer 5010 (Germany, 1990). Detection of IgG/IgM antibodies against *T. gondii* antigens from CSF and plasma were carried out using *T. gondii* antigen coated enzyme linked immunosorbent assay (ELISA) test kits (10). All ELISA tests were carried out in duplicate and averages of the optical density were taken for the results. Where

there are indeterminate results, the tests were repeated. Human immunodeficiency virus (HIV) testing was performed using HIV-1/2 Uni-Form II Ag/Ab Micro-elisa system bio-Merieux. In parallel, test for Brucela IgM and IgG antibody was done using Brucela Alfa Scientific Designs *Brucella* IgM and IgG test strip flow assays [(Organon Teknika, Ltd. (Dublin, Ireland)] respectively that were kindly supplied by Dr H. Smits kit, Holland. Three Hundred μl of whole blood samples were processed immediately within 2 hours of collection for staining with IgG1/IgG2a (isotype control), CD45/14 (leucogate), CD4/CD8 using fluorochrome labeled mouse monoclonal antibodies (Becton Dickinson, USA) following manufacturer's instructions and data was acquired and analyzed using FACScan machine (Becton Dickinson, USA).

Statistical Analysis

Stata version 7 software was used and a two by two table was done to compare different variables. Presence of statistical significance among the different variables was determined using Chi-square test and a p-value < 0.05 was considered statistically significant.

RESULTS

Forty eight of the fifty one plasma samples and 40/52 CSF samples were found to be HIV positive. Clinically, nine of the 53 patients had signs and symptoms of cryptococcal meningitis, 5 had pyogenic meningitis, 2 had pulmonary TB, 2 had central nervous system toxoplasmosis and 6 had TB meningitis. Forty seven samples have shown *M. tuberculosis* IS6110 genes amplification with INS1/2 and IS5/6 primer pairs at 245 and 980 bps, respectively (Figure 1A). Housekeeping gene of the respective CSF samples was also amplified at 268 bp as visualized using agarose gel electrophoresis (Figure 1B).

Figure 1A. 245 bp PCR amplicons of IS6110 genes; Lane 1 represents a 100 bp DNA ladder; Lane 2-6 represent CSF samples from patients; Lane 7 represents a known positive control and Lane 8 is non-template (negative) control

Figure 1B. 268 bp PCR amplicons of beta globin gene; Lane 1 represents a 100 bp DNA ladder; Lane 2-5 represent CSF samples from patients; Lane 6 is non-template (negative) control and Lane 7 represents a known positive control.

Figure 1. PCR amplification of IS6110 genes of *Mycobacterium tuberculosis* (A) and beta globin gene (B), collected from cerebrospinal fluid (CSF) of patients with chronic meningitis
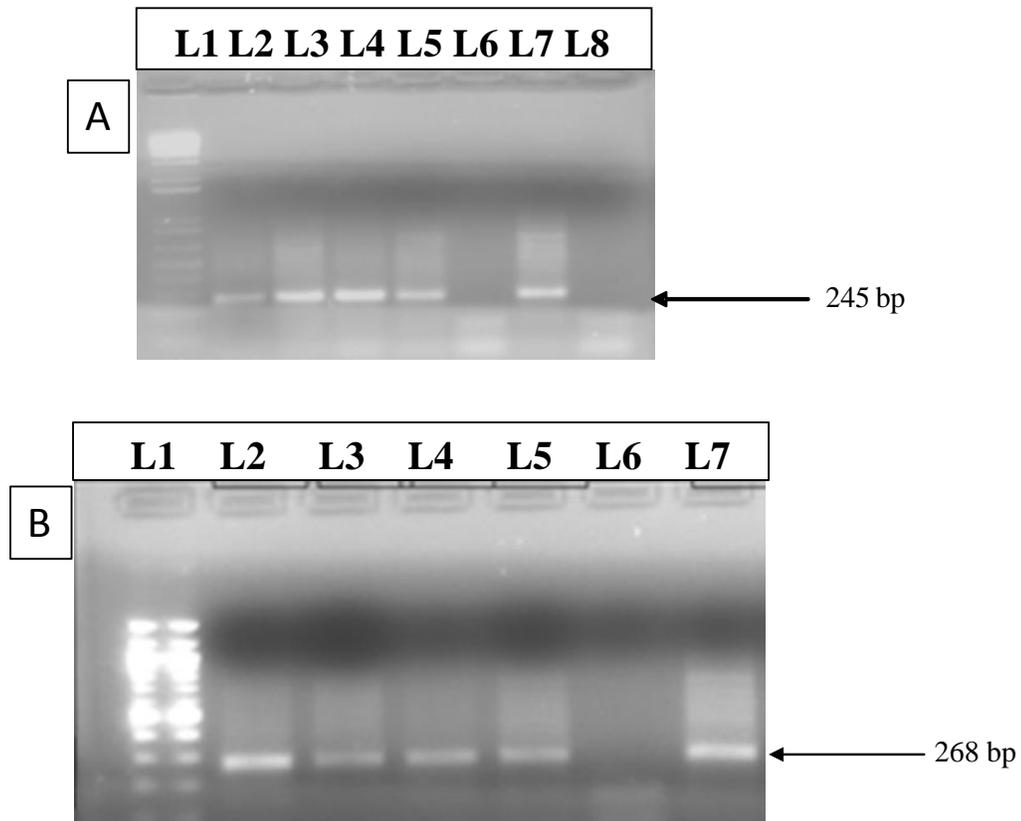
Of the PCR positive samples, only 1 was AFB (acid fast bacilli) positive from direct microscopy of CSF and 2 were culture positive. Although only nine were clinically diagnosed with cryptococcal meningitis, 18 were found positive by cryptococcal latex antigen detection test. The antigen detection test also picked four samples as positive that were negative by culture. Both IgM and IgG anti-Brucella antibodies were also detected in the plasma sample

and only IgG was detected in the CSF of one of the study participants. Eighteen of the fifty one plasma and 34 of the 52 CSF have showed IgG antibody positivity for *Toxoplasma gondii* antigens, while none of the samples showed positive result for IgM antibody. Mixed infections with *M. tuberculosis*, *C. neoformans, T. gondii, N. meningitidis* and HIV were detected in immunocompromised patients (Table 1). Among these patients one third of the HIV sero-positives, co-infected with *Cryptococcus neoformans* capsular antigen were having ≤ 0.1:1 CD4 to CD8 ratio (Table 2).

Table 1. Multiple infections detected in the cerebrospinal fluid of HIV positive study participants with <0.1 to 1 CD4 to CD8 ratio, recruited at Tikur Anbessa Teaching Hospital and Ye'huleshet Clinic between 2003-2004

Diagnosed multiple infections	Frequency of patients diagnosed
Cryptococcus neoformans	
Toxoplasma gondii*	6/52 (11.5%)
Mycobacterium tuberculosis	
HIV	
Cryptococcus neoformans	
Mycobacterium tuberculosis	2/52 (3.8%)
Nisseria Meningitidis	
HIV	

*Detection of *T. gondii* was measured in terms of IgG and not IgM

Table 2. CD4/CD8 ratio vs. *C. neoformans* antigen, *T. gondii* IgG antibody and *M. tuberculosis* IS6110 gene PCR positivity in the cerebrospinal fluid of chronic meningitis patients recruited at Tikur Anbessa Teaching Hospital and Ye'huleshet Clinic between 2003

Diagnosis	CD4/CD8 ratio*		
	≤ 0.1:1	1:1	≥ 1:1
***Cryptococcus neoformans* antigen**			
Positive	15/36 (41.7%)	-	-
Negative	21/36 (58.3%)	5/5	3/3
***Toxoplasma gondii* IgG**			
Positive	14/36 (38.9%)	2/5	1/3
Negative	22/36 (61.1%)	3/5	2/3
***M. tuberculosis* PCR status for IS6110 gene**			
Positive	31/36 (86.1%)	4/5	3/3
Negative	2/36 (5.6%)	1/5	-

and 2004

*CD4/CD8 ratio was done for 9 study participants only

DISCUSSION

The finding of mixed infection with the various infectious agents tested, were mainly manifested in the immunocompromised patients, a result in line with other studies (13) especially with central nervous system cryptococcosis (12). Especially our observation of *Cryptococcal meningitis* in immunocompromised individuals was similar with other studies done elsewhere (6). *Trepanoema palladium* and Brucella antigens (also known to be one of the etiologic agents of chronic meningitis) in the CSF were rare episodes that were seen only in two study participants. However, there was no acute infection with *T. gondii* observed in our study participants. In this study, fever was prominent among patients suffering from cryptococcal meningitis and with CSF HIV positive status ($P < 0.05$). As expected from such patients, the CSF to blood glucose ratio was < 0.6 in 18 patients. No discrepancy of result was found in the immune-assays of CSF and plasma i.e., infectious agents diagnosed positive in the CSF were also positive in the plasma. While no positive result of any of the studied infectious agents was found to be positive in the CSF while being negative in the plasma.

The trend of low CD4 to CD8 ratio, a risk to be exposed to opportunistic infections, was revealed in certain proportion of patients co-infected with *Cryptococcus neoformans* and HIV, though significant association was not obtained due to the smaller sample size used in this study. Such high finding of *Cryptococcal meningitis* in immunocompromised individuals from our study is in line with other studies (6). A high index of suspicion and improved diagnostic procedures and management are needed not to miss treatable conditions in patients having clinical diagnosis of chronic meningitis.

In conclusion, our study indicates that *C. neoformans i*s the major etiologic agent followed by *M. tuberculosis* in causing chronic meningitis. Polymerase chain reaction showed very high percentage of *M. tuberculosis* DNA amplification which may partly be due to entry of DNA to CSF through ruptured blood brain barrier. Clinical diagnosis results have also showed presence of *M. tuberculosis* in certain proportion of the studied patients. The trend of our finding shows that increased attention should be given to *C. neoformans* as the major etiologic agent and *M. tuberculosis* as the secondary etiologic agent of chronic meningitis during the management of immunocompromised adult patients who are clinically diagnosed for chronic meningitis.

ACKNOWLEDGEMENTS

REFERENCES

1. Davis LE. (2008). Acute and recurrent viral meningitis. *Curr Treat Options Neurol.* 10: 168-177.

2. Ginsberg L and Kidd D. (2008). Chronic And recurrent meningitis. *Practical Neurology.* 8: 348-361.

3. Joseph Z., Kelly B. (2013). Chronic and subacute meningitis. *AAN.* 18: 1290-1318.

4. Hildebrand J, Aoun M. (2003). Chronic meningitis: still a diagnostic challenge. *J Neurol.* 250: 653-60

5. Colombe B, Derradji M, Bosseray A, Massot C, Debru JL. (2003). Chronic meningitis: etiologies, diagnosis and treatment. *Rev Med Interne.* 24:24-33

6. Zaidi AK., Thaver D., Ali SA., Khan TA. (2009). Pathogens associated with sepsis in newborns and young infants in developing countries. *Pediatr Infect Dis J.* 28 (1 Supplement): S10-8.

7. Gallant JE, Moore RD, Chaisson RE. (1994). Prophylaxis for opportunistic infections in patients with HIV infection. *Ann Intern.* 120:932-44

8. Roullet E. (1999). Opportunistic infection of the central nervous system during HIV-1 infection (emphasis on cytomegalovirus disease. *J Neurol.* 246: 237-43

9. Ermak TN, Kravchenko AV, Iurin OG, Pokrovskii VV. (1999). The structure of the secondary diseases in HIV-infected patients in Russia. *ZH-Mikrobiol-Epidemiol-Immunobiol.* **1:** 80-2

10. Bicanic T, Harrison TS. (2005). Cryptococcal meningitis. *Br Med Bull.* 18: 99-118

11. Van Soolingen, D. (1998). Utility of molecular epidemiology of tuberculosis. *Eur. Respir. J.*11**:**795-797

12. Montoya JC, Recuisado G, Sombrero L. (1997). Detection of mycobacterium tuberculosis in cerebrospinal fluid of smear-negative tuberculous meningitis using polymerase chain reaction with nested amplification. *Phil J Microbiol Infect Dis*. 26:4

13. Rothschild BM., Martin LD, Lev G, Bercovier H, Kahila BG, Greenblatt C. *et al.,* (2001). *Mycobacterium tuberculosis* Complex DNA from an Extinct Bison Dated 17,000 Years before the Present. *Clin Infect Dis.* 33:305–11

14. Portillo PD, Thomas MC, Martínez E, Marañón C, Valladares B, Patarroyo ME, *et al.,* (1996). Multiprimer PCR system for differential identification of mycobacteria in clinical samples. *J Clin Microbiol.* 34: 324–328

15. Guebre-Xabier M, Nurilign A, Gebre-Hiwot A, Hailu A, Sissay Y, Getachew E, *et al.,* (1993). Sero-epidemiological survey of *Toxoplasma gondii* infection in Ethiopia. *EMJ.* 31**:** 201-8

16. Yeh TH, Lin SK, Lee MH, Chang WK, Wu JH, Ryu SJ. (1997). Mixed meningococcal and tuberculous meningitis. *J Formos Med Assoc*.; 96:461-4

17. Wantanabe H, Inukai A, Doyu M, Sobue G. (2000). Central nervous system Cryptococcosis with idiopathic CD4+ T lymphocytopenia. *Rinsho Shinkeigaku*. 40: 249-53