Molecular Characterization of *Mycobacterium tuberculosis* Isolated from HIV Patients in West Darfur State-Sudan

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Abstract

The aim of this study was the Characterization of Mycobacterium TB isolated from HIV/AIDS patients and was conducted in West Darfur State during February 2013 through July 2014.The study samples (sputum) have been collected from two groups : people living with HIV/AIDS (PLWHA) (N=158) and TB positive patients that were negative for HIV(N=200) as control samples .The study samples have been stained by ZN stain and investigated using the microscope ,six of the (PLWHA) was found to be positive for AFB, of which (66.7%) were males and(33.3%) were females , the most affected age group was 31-40 years (50%) , followed by 41-50 years (33.3%) which indicate negative impacts from the economical and social point of view.. All ZN stain positive samples (N=206) plus (12) ZN negative samples from PLWHA were subjected to DNA extraction by the modified Guanidine Chloride Method. The samples then processed by PCR technique which confirmed the results of the microscopy. The study concluded that the prevalence of TB among (PLWHA) in the study area is low. Further comprehensive studies applying advanced molecular techniques were highly recommended nationally and in the study area as well.

Keywords: Tuberculosis, HIV/TB Co-Infection, West-Darfur, PCR.

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Introduction

In the words of the WHO, HIV infection is "fueling the tuberculosis epidemic" as it is now the most important predisposing factor for the development of active tuberculosis. A non – immuno-compromised person who has overcome primary tuberculosis infection has about a 5 percent chance of developing post – primary tuberculosis later in life. In a person infected with both M. tuberculosis and HIV, the risk of developing active tuberculosis rises to around 8 percent each year – a greatly increased risk. Furthermore, the disease process which usually takes years or even decades to develop is telescoped down to just a few months. Little wonder, therefore, that co – infection with M. tuberculosis and HIV has been termed "The cursed duet" and that in addition to using the term "apocalyptic" the WHO has stated that " HIV and TB co – infection represents an unprecedented global public health crisis (Pratt et al. ,2005) . Today it is known that TB and HIV/AIDS are the two of the world's major pandemics. The pandemic of HIV/ AIDS opened a new chapter on the role of mycobacteria in causing human disease in both the developed and less – developed world. The number of people co – infected with TB and HIV already stands of over 10 million and is expected to increase dramatically over the next 10 years. To make matters worse, global rates of MDR – TB are also on the rise. MDR – TB is very complicated and difficult to treat, very expensive to treat and often fatal (Sharma, 2004). Tuberculosis is particularly insidious problem to those who have AIDS. Unlike most other tuberculosis patients, those with HIV usually develop tuberculosis in the lymph nodes, bones, liver, and numerous other organs (Pommerville ,2004).

Infection with HIV -1 has emerged as the single strongest risk factor for development of active TB. This may be the result of number of different processes and underlying immunological mechanisms. The fact that certain species of innate immune function are

impaired by HIV infection might potentially increase host susceptibility to M. tuberculosis infection following exposure. (Schaaf and Zumla ,2009)

Globally, approximately 9% of all new TB cases in adults are attributable to HIV infection. The proportion is much greater in sub–Saharan Africa. The close links between HIV and TB, and the potential for both diseases overwhelm health–care funding in resource. Poor nations have been recognized with the promotion of programs that link detection and treatment of TB with detection and treatment of HIV ((Boon et al. ,2006)).

Collaborative TB/HIV activities are essential to ensure that HIV-positive TB patients are identified and treated appropriately, and to prevent TB in HIV-positive people. These activities include establishing mechanisms for collaboration between TB and HIV programs (Friede,2004).

Sudan has the highest pulmonary TB incidence among the Eastern Mediterranean countries, as reported by the World Health Organization. Active TB disease is the commonest opportunistic infection amongst HIV-infected individuals; in OMACU (TB/HIV centre in Khartoum), the prevalence of TB amongst HIV patients is15% in 2008 (SNAP,2002).

Materials and Methods

Study Area and Popultion

This study was conducted in West Darfur State, Western Sudan, during February 2013 through July 2014 in two groups , People Living with HIV and AIDS (**PLWHA**) and **TB** patients who were negative for HIV, as a control group which was objected to characterize *Mycobacterium tuberculosis* bacilli isolated from HIV/AIDS patients .

One hundered fifty eghit sputum samples were collected from (PLWHA) patients presented at VCT Center (attached to Algeneina Hospital) and at the Center of (PLWHA) Association in Algeneina . Other AFB positive sputum samples (n= 200) were collected from patients suffering from pulmonary TB presented at Algeneina TB Center in Algeneina Hospital.

ZN Stain (Smear Microscopy)

For suspected cases of pulmonary TB, two sputum samples from each subject were collected into a well-labeled wide mouth container covered with lid. Diagnosis of TB was based on the (WHO) guidelines for TB diagnosis by microscopy. Two consecutive sputum samples (spot - morning) were collected and examined for the presence of Acid Fast Bacilli (AFB) using the standard Ziehl-Neelsen method. The sputum evenly spread over the central area of the slide , covered with 0.3% Ziehl's carbol fuchsin working solution and heated until vapour starts to rise , leave for 5 min., the slides then gently rinsed with tap water, the slides were covered with 25% sulfuric acid solution and allowed to stand for 2 min., the acid washed away with water, The slides were individually covered with 0.3% methylene blue counterstaining solution , allowed to stand for 1 min..Fnally, drain water off the slides, allowed to air dry and seen under the microscope . The positive sputum samples plus some selected negative samples were stored at -20°C and transported to Khartoum for further advanced molecular techniques.

DNA Isolation

Sputum samples from all ZN stain positive TB patients (N= 200 patients) and from PLWHA (N= 6) plus selected negative TB samples (N=12)from (PLWHA) were subjected to DNA extraction using Guanidine Chloride Method. Sputum samples were taken in tubes , equal volume of sodium hydroxide (4%) was added, mixed well and centrifuged for 15min-at 4000rpm, the supernatant was discarded , 2000 µl of WBCs lysis buffer , 10 µl of proteinase K,300 µl of ammonium acetate and 1000 µl of guanidine were added , 2000 µl of chloroform was added after incubation at 65 ^{o}C for 2 hours and centrifuged at 3000rpm for 10 min., 10 ml of 95% cold ethanol was added and incubated overnight at -20^{o}C, the tubes were centrifugated at 3000rpm for 10 minutes and the supernatant was discarded off, the tubes then dried, 4 ml of 70% ethanol was added to the tubes and centrifuged at 3000rpm for 10 min. , the supernatant was discarded, the pellet was allowed to dry for 15min. and 50 µl of distilled water was added, the extracted DNA was stored at -20^{o}C till used.

DNA Amplification By PCR

Oligonucleotides to amplify 123 bp fragment of the IS6110 sequence of M.tuberculosis complex were used as primers (eurofins Genomics) in PCR technique with the following sequence:

IS6110-**Forward 5′-CCTGCGAGCGTAGGCGTCGG-3′**

IS6110-**Reverse- 5′CTCGTCCAGCGCCGCTTCGG-3′**

A template DNA , primers, Maxime PCR PreMixtubes (i-Taq) (iNtRONBIOTECHNOLOGY-Korea) which contains (i-TaqTMDNA Polymerase, dNTP mixture and reaction buffer) and distilled water were prepared (Table 1). The PCR was briefly performed under the following conditions , initial denaturation 94℃ for 5min., denaturation at 94℃ for 30 sec., annealing at 50-65℃ for 30 sec., extension at 65-72℃ for 30 sec. with final extension at 72℃ for 7 min. The samples then loaded on 2.0% ag`arose gel and electrophoresis was performed, DNA bands was visualized by UV light.

RESULTS

Of the total 158 PLWHA patients tested for TB in this study 6/158 (3.8%) were found smear positive by microscopic examination after staining by ZN stain method (Figure1).

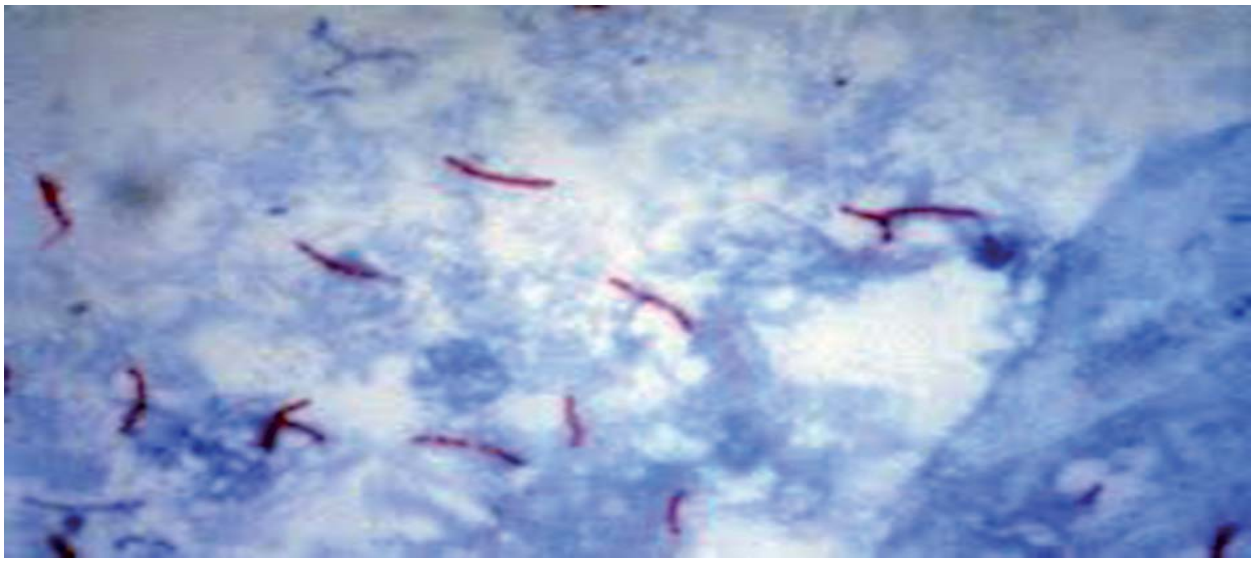

Figure 1. The AFB in Smear Microscopy by ZN Stain.

The prevalence rate of TB among HIV infected patients in the study area was significantly higher in males, 4/6 (66.7%) compared with females, 2/6 (33.3%). (Figure 2).

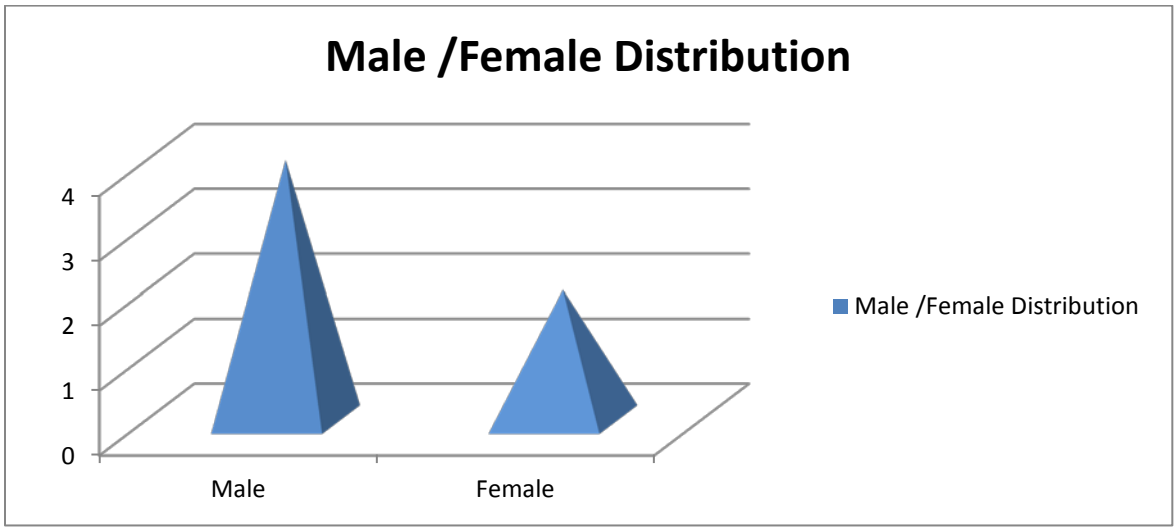

Figure 2. Male Female Distribution among TB Positive PLWHA in The State.

The TB prevalence among PLWHA was observed to be higher in the age group 31-40 years (50%) and 41-50 years (33.3%) compared to younger age groups of 21-30 years (16.7%).(Table 1).

(Table 1) Age Group Distributions among The Study Population.

Age group	Number	percentage
20-30	1	16.7
31- 40	3	50.0
41-50	2	33.3
51-60	0	0
Total	6	100

Of The 200 smear positive sputum samples taken from the TB patients whom were negative for HIV infection as the control samples , (90 %) 180/200 are found PCR positive .

All the ZN (microscopy) negative sputum samples (N=12) taken from (PLWHA) for comparison bettween microscopy and molecular techniques were gave negative Results by PCR.

The method used for DNA extraction from the sputum samples in this study (Modified Guanidinium Isothiocyanate Method) has given good results in the PCR technique indicating that it has no noticeable PCR inhibitors and it is suitable for *M. tuberculosis* DNA extraction .

PCR technique which used IS6110 primer for detecting *M. tuberculosis* in this study ,showed clear obvious bands on gel electrophoresis revealing a good PCR product. (Figure 3).

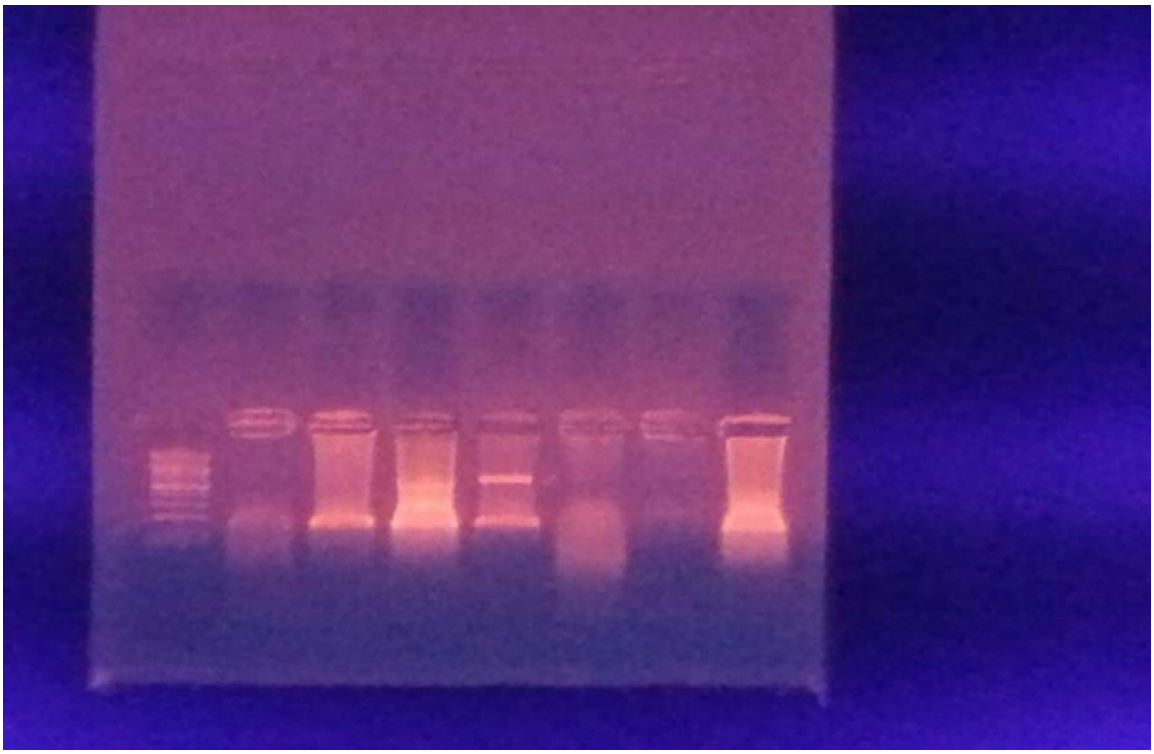

Figure (3) Bands on Gel Electrophoresis after PCR.

Most of the TB positive PLWHA were from the VCT Center 5/6 (83 %) and only one 1/6 (17%) was from the Center of (PLWHA) Association in the state showing relatively good health care given to the members suffering from HIV infection by governmental and non- governmental

agencies in the State and the excellent collaboration between State TB and HIV programs. (Figure 4) .

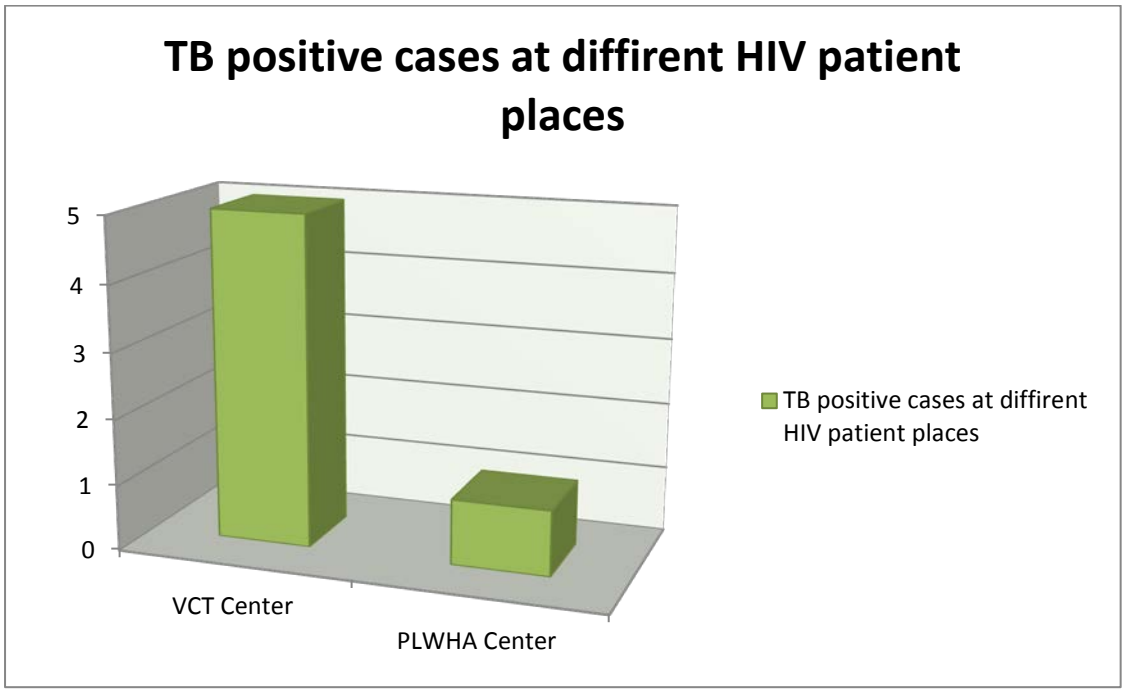

Figure 4 TB Positive Cases at The Different HIV Infected Patients Study Centers.

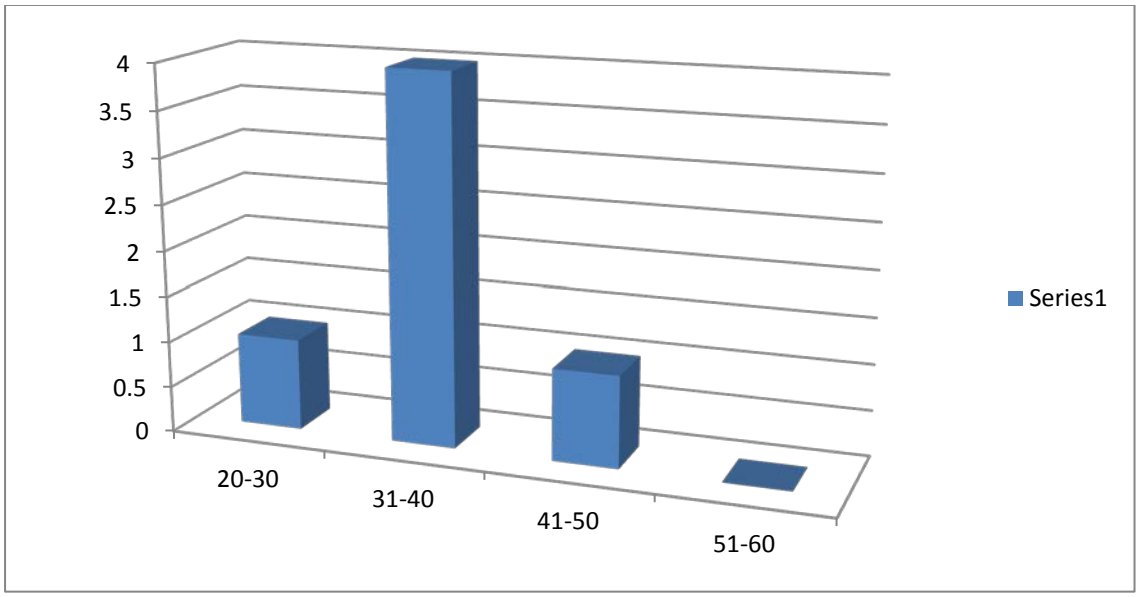

Figure 5. Age Distribution among PLWHA Co-Infected with HIV/ TB.

Discussion

The results showed by this study were consistent with other studies conducted in Accra city – Ghana of (3.6%) prevalence of TB among HIV sero-positive patients (Essiam, 2013). The prevalence rate of TB in this study was less than the results of a study carried In Uganda to evaluate the Prevalence, incidence and mortality associated with tuberculosis in HIV-infected patients initiating antiretroviral therapy in Rural Uganda of (7.2 %) TB prevalence (Moore et al., 2007). However, the prevalence rate observed in this study was to a significant extent lower compared to other previous studies conducted in India (49.2%) prevalence of TB among HIV-positive subjects in Gujarat –India (Ghiya et al., 2009) which reflect a very high TB prevalence among PLWHA in Gujarat region in India. Our Study results were also not agreed with other research findings from Rural Cambodia found by Cain and others with prevalence rate of (38.0 %) TB in HIV infected patients (Cain et al., 2007) and with that carried by Mihir and his collogues among HIV Sero-positives Attending a Counseling Center in Kolkata-India (33.0%) prevalence (Mihir et al .,2011). The variations in this study results and the above mentioned study findings is multifactorial, , may be according to the different methodologies carried by the studies ,or the diagnostic techniques used whether they are conventional or advanced beside the different study populations factors which have important roles.

However, most of these studies included all forms of tuberculosis, while the present study focused on pulmonary tuberculosis only. The difference could be due to the difference in the general HIV prevalence rates reported in the countries in which the researches are carried. Part of the differences between the prevalence rate in this study and the other studies may be due to differences in inclusion and exclusion criteria. In turn, the diagnosis of TB in these patients (PLWHA) can be problematic, depending on the degree of immunosuppression at the time of diagnosis.. The current prevalence rates of TB among PLWHA in West Darfur State were still low when compared with prevalence rates of a study carried in Northern Tanzania in 2008 of (8.2%) entitled Prevalence among People Living With HIV/AIDS Attending Care and Treatment in Rural Northern Tanzania (Ngowi et al ., 2008). This discrepancy would be probably attributed to the type of study, the study time and the study area. whoever , the study findings are more closer to a research results showed by Sudre and others in Switzerland (3.4%) prevalence rate of tuberculosis among HIV infected patients (Sudre et al .,2002) and quiet

similar to prevalence rates observed in a study carried in Rural area of Maharashtra-India (3.4%) aimed to estimate the Prevalence of Tuberculosis in the Rural area of Maharashtra among HIV infected Persons(Tamboli et al.,2010) .The reduction in the prevalence of TB in HIV infected patients may be due to the introduction of HAART which is found to has remarkable changes towards improving the management of HIV patients. We believe that, the rate of pulmonary tuberculosis could be higher if new diagnostics, particularly Xpert MTB/RIF was performed routinely for mycobacterium tuberculosis diagnosis in these studies. In a study conducted in Islamic Republic of Iran, the rate of pulmonary tuberculosis in HIV-infected patients was found to be 11.7 % and the remaining cases were extra pulmonary or disseminated tuberculosis (Mansoori et al .,2002). In another study conducted in Nigeria during 2013 intended to find the currently factors affecting treatment outcomes of tuberculosis in a Tertiary Health Center in SouthWestern Nigeria, the rate of pulmonary tuberculosis among HIV-infected patients was 20.0% (Babatunde et al.,2013) which is high compared to the present prevalence of this study . WHO guidelines call for culture to be performed on sputum smear-negative HIV-infected patients with a clinical suspicion of TB; however, these tests are more expensive to perform and require upgraded infrastructure and skilled laboratory staff (Parsons et al ., 2011)and it is difficult to be applied in the study area because of the limited resources . The clinical care and close follow-up provided for HIV infected patients in the VCT and PLWHA centers in the study area might have further reduced transmission and might explain the lower prevalence of TB among HIV-infected patients, compared to HIV-negative patients. Of note, TB among the HIV-infected population was at least partly due to transmissions from HIV negative individuals.. While the scale of the HIV-TB syndemic seems daunting, the application of existing knowledge and techniques for the diagnosis, treatment, and prevention of TB can make an impact. Further progress will require advances in our understanding of the dual biology of HIV and TB co-infection as well as a better understanding of the interactions of *M. tuberculosis* with HIV-infected and -uninfected humans and their innate and adaptive immune systems.

The PCR results obtained in this study ,The DNA sequence most frequently used to detect M. tuberculosis has been the insertion element IS6110 and it has been used successfully by Osman and others is a study entitled Improved Detection of *Mycobacterium tuberculosis* in Sudanese Children by use of Insertion Sequence IS6110 (20). Moreover ,IS6110 gene is used as a target by the majority of the investigators performing PCR based diagnosis of TB , the principal

reason for using IS6110 is because it is considered to be a good target for application as this insertion sequence is found in almost all members in highly copy number in most strains of *Mycobacterium tuberculosis* complex, which was thought to confer high sensitivity, as reported by Gasmel seed and collogues in their work which carried in Wad Medani Hospital – Sudan, during 2009-2012 for the assessment of different samples for molecular diagnosis of etra-pulmonary tuberculosis (Gasmelseed et al .,2014) and this is why the present study used this IS6110 gene , and the good results achieved by PCR and DNA sequencing techniques may highly be due its use .

Concerning the 90% positivity of the study control samples collected from TB patients negative for HIV when performed by PCR technique in this study ,the results are so close to that reported by El Dawi and his follows in a study which aimed to assess and evaluate the PCR-amplified IS6110 insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan, of 88.5% sensitivity and 98.6% specificity for IS*6110*-based PCR for identification of *M. tuberculosis* isolates from Khartoum(El Dawi et al.,2004). Based on the results of this study, it is clear that the techniques used the our study were reliable and can be used for diagnosis and characterization of *Mycobacterium* isolates.

The most infected sex group in this study were males and this is close to results showed by Abdallah and others at Kasala State in Sudan, aiming to investigate the sero-prevalence of HIV among TB patients were reported in Kassala Teaching Hospital, during January 2008– through December 2010 (Abdallah et al., 2012). Whether, and the extent to which, identifying fewer women with TB globally is due to sex (as a biological determinant) or gender (as a socio-cultural determinant influencing access to TB care) have been issues for discussion and debate. While some attribute it to barriers women may face in accessing TB care, others ascribe it to the natural epidemiology of the disease (Ottmani and Uplekar., 2008). in the study area community, coughing and spitting up sputum are not socially accepted, especially among women, this cultural belief prevents women from deeply coughing and spitting up a good sputum sample, therefore, the probability of finding AFB in the sputum produced by women is likely to be lower than by men.

The TB prevalence among PLWHA in the study area was found higher in the age groups 31-40 years and 41-50 years of age(Fig.5) .This age distribution of TB cases in the study population is in compliance with the global and national trends that the majority of TB cases

are in their productive ages ,and this may also reflect the economical burden . The official prevalence of TB infection in Sudan is about 209 cases per 100,000 of the population in 2009(SNTCP.,2011). High incidence ranks Sudan among the high prevalence countries for TB in the Eastern Mediterranean Region and accounts for 14.6% of the total TB burden (Khalid et al., 2015). However, the prevalence rates of TB/ HIV co-infection in West Darfur State in the years 2013 and 2014 was 3.0 % and 4.5% respectively (W.D.TB.records ,2015) which is very closer to the results of the present study . Thus, this study conducted in public DOTS clinic and (PLWHA) center of the biggest city in West Darfur State , would probably give a representative picture of tuberculosis among HIV co-infection in the State .

Conclusions

The study showed that 3.8% of people living with HIV/AIDS were also co- infected with tuberculosis using conventional methods and confirmed by molecular techniques.. This prevalence of HIV/TB co-infection in the state is low compared to the global data.More strategic preventive measures that enhance body immunity among HIV patients are highly needed as early as possible before they develop active tuberculosis. Further comprehensive studies are required to fully ascertain the extent of the problem in the whole country applying specific and rapid molecular diagnostic technologies.

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