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Diagnostic Perfofmance of Ziehl-Neelsen, Flourescent Auramine O Stains and Genexpert for the Detection of Mycobacterium Tuberculosis

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Abstract: *Tuberculosis (TB), one of the oldest known diseases and still a major cause of mortality today, many other organ systems such as the bone and central nervous system are manifested and affected by the disease. It is caused by a closely-related group of organisms, all of which form the Mycobacterium tuberculosis complex. These organisms include M. tuberculosis, M. bovis, M. africanum, M. microfti and M. canett. A cross sectional comparative study was conducted to assess the efficacy of the different diagnostic method of pulmonary tuberculosis of sputum samples from patients suspected of pulmonary tuberculosis. The study was conducted in the period from Feb 2015 to May 2016.*

Early morning sputum samples were collected in clean, sterile, leak proof, wide mouth containers and sent to the laboratory without delay.

The processing of the sample carried out according to a bio-safety considerations. Each sample was subjected to Ziehl-Neelsen (ZN) staining and fluorescent Auramine-O (AO) staining, and then each sample was run on Gene Xpert, new clean, unscratched slide was labeled at one end with the laboratory number. Mucopurulent portion was used for smear preparation. An appropriate portion of the specimen transferred to the slide using a wooden-stick and smeared over an area of approximately 2 by 3 cm. A new slide for each specimen used. The smears allowed to air-dry for 15 minutes then fixed by passing over the flame 3 to 5 times for 3 to 4 seconds.

Two hundred and forty one sputum specimens were examined by ZN staining technique , 52 (21.7%) of them were showed the characteristic of AFB appearance of serpentine cords under oil immersion field. Sixty two out of 241 (25.7%) sputum specimens using Fluorescent(AO) staining procedure showed the typical characteristic of AFB bacteria. Sixty seven out of 241(27.8%) were mycobacterium tuberculosis complex.

In this study out of 241 specimens 67 (27.8%) were shown MTB detected on Gene Xpert while 52 (21.5%) were AFB positive by ZN staining and 62 (25.7%) were AFB positive when Fluorescent staining was used.

The study concluded that as compared to AFB microscopy, Gene Xpert is more sensitive and specific not only for acid fast bacilli (AFB) detection but also for rifampacin (RIF) resistance. It also helps to avoid injudicious use of anti tuberculosis drugs.

Keywords: *Auramine O , Flourescent , Genexpert , Tuberculosis, Ziehl-Neelsen.*

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I. Introduction

Tuberculosis (TB), one of the oldest known diseases and still a major cause of mortality today, many other organ systems such as the bone and central nervous system are manifested and affected by the disease. It is caused by a closely-related group of organisms, all of which form the *Mycobacterium tuberculosis* complex. These organisms include *M. tuberculosis, M. bovis, M. africanum, M. microfti and M. canett* [(1)].

Tuberculosis is a bacterial highly chronic prevalent infectious disease caused by an intracellular aerobic bacteria known as mycobacterium tuberculosis because of this characteristic it prefers tissues which are always in contact with high oxygen levels as in lung. Transmission of disease is by inhaling the bacillus, transmitted by tiny droplets of saliva, then individual develop the disease depending on his immunological status. After taking up residence in the lung, M. tuberculosis can disseminate to any part of the organ [(1)]. Many infected peoples develop no symptoms because the inhaled bacteria lives in inactive form inside the lung and it will transform into active form when the patient immune system became week.

The appearance of drug-resistant strains of TB and increase among HIV infections and also among homeless people in poor environment in addition to malnutrition greatly increases the risk of developing

tuberculosis and accelerates its progress. In 2003, WHO estimated that there were 8.8 million new cases of tuberculosis with 1.75 million deaths while in 2010, also estimated 8.8 million new cases and 1.5 million associated deaths occurred, mostly in developing countries. Mortality rates are highest among children and young adults so rapid diagnosis and control is important. The most important tool for the control of tuberculosis is the microbiological diagnosis which is consist of conventional methods (acid-fast microscopy, culture, biochemical identification, anti-tuberculosis drug-susceptibility test) and modern molecular techniques. The rapid detection of *Mycobacteria* in clinical specimens is essential for the early diagnosis and treatment of patients.

II. Materials And Methods

Study design

A cross sectional comparative study was conducted to assess the efficacy of the different diagnostic method of pulmonary tuberculosis of sputum samples from patients suspected of pulmonary tuberculosis. The study was conducted in the period from Feb 2015 to May 2016.

Method

Early morning sputum samples were collected in clean, sterile, leak proof, wide mouth containers and sent to the laboratory without delay [(2)] .

The processing of the sample carried out according to a bio-safety considerations. Each sample was subjected to Ziehl-Neelsen (ZN) staining and fluorescent Auramine-O (AO) staining, [(2)] and then each sample was run on Gene Xpert, new clean, unscratched slide was labeled at one end with the laboratory number. Mucopurulent portion was used for smear preparation. An appropriate portion of the specimen transferred to the slide using a wooden-stick and smeared over an area of approximately 2 by 3 cm. A new slide for each specimen used. The smears allowed to air-dry for 15 minutes then fixed by passing over the flame 3 to 5 times for 3 to 4 seconds.

Staining

Procedure of Ziehl-Neelsen Stain

- The smears of the specimen were fixed over the glass slide by heating.
- Carbol fuschin was poured over smear and heated gently until fumes appeared. and allowed to stand for 5 minutes, then washed off with water.
- Sulphuric acid (20%) was poured wait for one minute and repeated this step until the slide appeared light pink in color. Washed off with water.
- Methylene blue, was added for two minutes, then washed with water
- Then dried in air and examined under oil immersion lens.

Procedure of fluorescent stain

- Filtrated auramine O was poured over smear and allowed it to stand for 15 minutes, then washed it off with water.
- Acid alcohol 3% was poured wait for five minute and Washed off with water.
- Methylene blue, was added for two minutes, then washed with water.
- Allowed it to air dry and then examined under fluorescent microscopy.

Standard Assay Procedure of Gene Xpert

Each Xpert MTB/RIF cartridge was labeled with the sample identity (case number). Transferred at least 0.5 mL or 1ml sample of the total resuspended pellet to a conical, screw-capped tube for the Xpert MTB/RIF using a transfer pipette sputum sample was liqufactioned and inactivated with 2:1 of sample reagent. Incubated for 10 minutes at room temperature, and then shacked the specimen vigorousliny 10 to 20 times or vortex for at least 10 seconds. Incubated the sample at room temperature for an additional 5 minutes. Started the test within 4 hours of adding the sample to the cartridge. Opened the cartridge lid, and then opened the sample container. Using the provided transfer pipette, aspirated the liquefied sample to the line on the pipette. Transferred 2ml of the sample into the sample chamber of the Xpert MTB/RIF cartridge. The sample was dispensed slowly to minimize the risk of aerosol formation. Closed the cartridge lid firmly. Loaded the cartridge into the Gene Xpert Dx instrument and started the test within 5 hours of preparing the cartridge. Turned on the GeneXpert instrument. GeneXpert Dx instrument, first turn on the GX Dx instrument, and then turned on the computer. The GeneXpert software was launched automatically. The Scanned Sample ID dialog box appeared. In the Sample ID box, typed the sample ID. The Scanned Cartridge Barcode dialog box appeared. Scanned the barcode on the Xpert MTB/RIF cartridge. The Create Test window appeared. Using the barcode information, the software automatically filled the boxes for the following fields: Selected Assay, Reagent Lot ID, Cartridge SN, and Expiration Date. Opened the instrument module door with the blinking green light and loaded the cartridge. The door was closed. The test started and the green light stops blinking. When the test finished, the light turns off. Waited until the system releases the door lock at the end of the run, then open the module door and remove the cartridge [(3)].

III. Results

Two hundred and forty seven sputum specimens were collected from patients suspected with pulmonary TB who presenting to the hospitals , six specimens were excluded due to errors or invalid results in GeneXpert interpretation. A total of 241 patients were included in this study, 141 (58.5%) of them were males and 100 (41.5%) were females. Female to male ratio of 1:1.41.Mean age of the patients was found to be 35.3±15.9. Most of the subjects (40.9%) were in 21-40 years of age. Two hundred and forty one sputum specimens were examined by ZN staining technique , 52 (21.7%) of them were showed the characteristic of AFB appearance of serpentine cords under oil immersion field. Sixty two out of 241 (25.7%) sputum specimens using Fluorescent(AO) staining procedure showed the typical characteristic of AFB bacteria.
Sixty seven out of 241(27.8%) were mycobacterium tuberculosis complex.

In this study out of 241 specimens 67 (27.8%) were shown MTB detected on Gene Xpert while 52 (21.5%) were AFB positive by ZN staining and 62 (25.7%) were AFB positive when Fluorescent staining was used. In table 1 a comparison between GeneXpert and ZN staining results was done , 50 (20.7) specimens were positive by both tests. Five specimens contained rifamapcin resistant strains,3 of them were smear positive while two were smear negative. Among the total number of specimens ,the positivity rate by both techniques (fluorescent stain (A-O) and Gene expert MTB/Rif) were 24%(58/241). Fifty eight out of 67 (86.6%) were positive by both methods , 4/62(6.4%) specimens showed smear positive (AFB) only by fluorescent (A-O) staining and was considered as Mycobacterium other than tuberculosis (MOTT) while nine (13.4%) specimens were positive by GeneXpert method but showed negative results by fluorescent (A-O) stain, 2 out nine(22.22%) specimens which MTB detected only by geneXpert showed rifamapcin resistant (Table 2) , when consider the total number of specimens ,the positivity rate by both techniques were 24%(58/241).

Forty eight (77.4%) specimens revealed typical characteristic of AFB by both procedures (ZN stain and FL (AO) stain). However 4 specimens (2.2%) were positive only by ZN staining . 14 specimens (22.6%) were positive by FL(AO) stain method but not ZN staining method (table 3). The distribution of mycobacterium tuberculosis infection (susceptible strains) among different age groups illustrated in Table 5 which reflected that the more prone population to tuberculosis concentrated in age ranged from 21 to 40 years (56.5%.), while table 6 illustrated the distribution of rifampacin resistant according to age which revealed that the population at risk of getting multidrug resistance was also in same ages ranged from 21- 40 years (80%).

Among the total study population five out 241 (2.07%) were rifampacin resistant detected by GeneXpert, three of the rifampacin resistant bacilli was AFB microscopically while the two other rifampacin resistance MTB were not detected microscopically and consider smear negative (table 1 and 2). The percentage of rifampacin resistance among the Tuberculosis patients which revealed smear positive was 3/58 (5.17%),while rifampacin resistance by GeneXpert for patients had smear negative represented 1.1% (2 /179) among the total number of smear negative specimens ,the total percentage of patients had rifampacin resistant by GeneXpert regard less of positivity of smear was 7.4% (5/67) (table 7).

In the present study the sensitivity and specificity of FL 89,2% and 97,7% and the sensitivity and specificity of ZN staining was 76.9% and 98.8% respectively when compared with geneXpert (table 8). the sensitivity and specificity of FL (AO) staining 92,8% and 92,5% respectively , the sensitivity and specificity of geneXpert 98.8% and 96.3% respectively when compared with ZN staining .(table 9).

IV. Figures And Tables

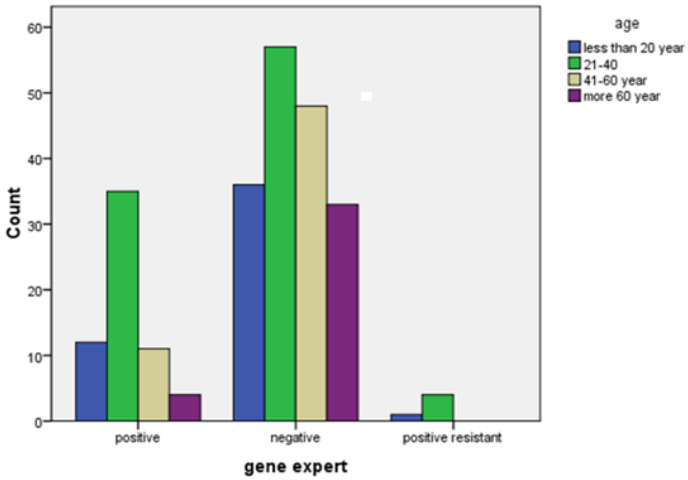

Figure 4.1 Distribution of MTB detected ,Rifamapcin not detected (sensitive) and Rifamapcin detected(resistance) correlated with age group.

P value =0,004.

Table 1. Comparison between ZN stain and Gene expert.

Gene expert / ZN stain	Positive	Negative	positive resistant	Total
Positive	(47) 75.8%	(2) 1.1%	(3) 60.0%	52(21.6%)
Negative	(15) 24.2%	(172) 98,9%	(2) 40%	189(78.4%)
Total	62(100%)	174(100%)	5(100%)	241(100%)

(P<0.000)

Table 2. Shows comparison between fluorescent stain (A-O) stain and Gene expert.

Gene expert / Fl stain	Positive	Negative	Positive resistant	Total
Positive	(55) 88.7%	(4) 2.3%	(3) 60.0%	62(25.7%)
Negative	(7) 11.3%	(170) 97.7%	(2) 40%	179(74.3%)
Total	62(100%)	174(100%)	5(100%)	241(100%)

(P<0.000)

Table 3. comparison between Ziehl-Neelsen and Fluorescent AO staining Result of smear examination by Ziehl-Neelsen and Fluorescent AO staining

FL stain / **ZN stain**	Positive	Negative	**Total**
Positive	(48) 77.4%	(4) 2.2%	52
Negative	(14) 22.6%	(175) 97.8%	189
Total	**62**	**179**	**241**

Method	**ZN stain** Positive	**FL stain** positive	**ZN +** **FL-**	**ZN -** **FL+**
NO	52	62	4	14
%	21.5%	25.7%	**2.2%**	**22.6%**

(P<0.000)

Table 4. Comparison between Gene expert, ZN staining and Fluorescent staining

Test	MTB detected (Positive)		MTB not detected (Negative)	
Gene expert	67	27.8%	174	72.1%
Fluorescent staining	62	25.7%	179	74.2%
ZN staining.	52	21.5%	189	78.4%

Gene expert	ZN staining smear.		Fluorescent staining smear	
	AFB +ve Positive	AFB –ve Negative	AFB + Positive	AFB –ve Negative
MTB +ve	50	17	58	9
MTB –ve	2	172	4	170
Total samples	52	189	62	179

(P<0.000)

Table 5. The distribution of Mycobacterium tuberculosis and susceptible strains to Rifampicin by GeneXpert according to age group.

Age	**MTB detected and Rifampcin not detected (Sensitive)**	
	NO	**Percentage %**
Less than 20 year	12	19.4%
21-40 year	35	56.5%
41-60 year	11	17.7%
More than 60 year	4	6.5%
Total	62	100%

(P<0.004)

Table 6. Distribution of Rifampacin resistance according to the age group.

Age	**MTB detected and Rifamapcin Resistant detected**	
	NO	**Percentage %**
Less than 20 year	1	20%
21-40 year	4	80%
41-60 year	0	0%

More than 60 year	0	0%
Total	5	100%

(P<0.000)

Table 7. Results of 'GeneXpert for detection of *Mycobatrium tuberculosis* and rifampacin resistant patterns.

RIF Resistance	MTB RESULTS		Total samples
	MTB DETECTED (+ve)	MTB Not DETECTED(-ve)	
RIF NOT DETECTED	62	179	241
RIF DETECTED	5	0	0
Total samples	67	179	241

(P<0.000)

62 out of 241 specimens were mycobacterium complex and were susceptible to rifamapcin while 5 specimens showed rifamapcin resistance.

The analytical sensitivity, specificity, of the method for the diagnosis of tuberculosis were calculated, by using the follow formulas; the Gene expert is standard test

$$\text{Sensitivity} = \frac{\text{True positive}}{(\text{True positive} + \text{false negative})} * 100$$

$$\text{Specificity} = \frac{\text{True negative}}{(\text{True negative} + \text{false positive})} * 100$$

Table 8. Sensitivity and specificity of ZN staining method and Fl (AO) staining method against Gene Xpert for the diagnosis of tuberculosis.

Factor	ZN stain smear	Fl stain smear
Sensitivity	**74.6%**	**86.6%**
Specificity	**98.8%**	**97.7%**

P value =0,000

sensitivity, specificity, of ZN staining method and Fl (AO) staining method for the diagnosis of tuberculosis when using Gene expert as standard method.

Table 9. Sensitivity and specificity of Gene expert method and Fl (AO) staining method against ZN staining for the diagnosis of tuberculosis

Factor	Gene expert	Fl stain smear
Sensitivity	**96.2%**	**92.3%**
Specificity	**96.1%**	**92.6%**

sensitivity, specificity, of Gene expert method and Fl (AO) staining method for the diagnosis of tuberculosis when using ZN staining as standard method

V. Discussion

Sudan was one of the largest countries in Africa, with regard to the surface area. The low national income and meager resources resulted in poor health services to the needy communities. The civil war and the displacement of population has complicated the health problems and helped the spread of infectious diseases in the different parts of the country. Although TB is known to be endemic in Sudan, little information is available about the burden of the disease, its epidemiology, or extent of drug resistance problem.

Early diagnosis of TB is necessary to disrupt the disease transmission chain. Although ZN smear positive patients are considered highly infectious and being focused by most of clinicians [(4)].

It is clearly TB patients in the current study have an age range between less than 20 to 50 years (75%) and this finding agreed with study by Murray et al (1990) who revealed that the most TB patients ,80% had ages less than 50 years. This is the case in most developing countries ,where as in Europe TB was more prevalence in elder people due to other factors such as diabetes mellitus and also among Immunocompromised patients.

A total of 241 patients were included in this study, 141 (58.5%) of them were males and 100 (41.5%) were females. Female to male ratio of 1:1.41. similar finding was reported by Munir *et al* (2014) who found female to male ratio of 1:1.08 and most of TB patients lie in the most productive age21-50 years (62.3%).

Smear positivity in the current study was found to be 21% for ZN stain, 25.7% for fluorescent (AO) stain and 27.8% when using GeneXpert among the total number of specimens , these finding were not in agreement with previous study which showed 67.5% for ZN and 77.4% for GeneXpert, another previous study

showed 53% by ZN and higher MTB detection by GeneXpert (82%) [(5)], the high variation in positivity rate between the present study and the previous studies may be attributed to inclusion of high suspicious in the previous studies in addition to the type of cases and high prevalence of TB in their setting ,in the present study all cases were new cases and not received any antituberculus drugs .

In comparison the finding of ZN staining and Fl staining the concordance results were obtained in The case detection rates for suspected patients with no treatment were 22% ZN, and 25. 8% Fl staining , 77.4% by both methods , 2.2% was detected as AFB by ZN staining only while 22.6 % was detected only by fluorescent staining, this is supported by Nissa 2015 which found out of 48 pulmonary samples 6(12.5%) were positive by ZN stain and 7 (14.6%) were detected by FL staining. Also Timalsina *et al* found 19.06%, 29.1% and 24.41% were found pulmonary tuberculosis positive by ZN, AO and culture respectively. The case detection rates for suspected patients with no treatment were 20%, 25.88% & 28.24%. Another study by Roma Goyal & Anil Kumar to screen Acid Fast Bacilli (AFB) by Z-N & Fluorescent staining methods. Positive samples detected by fluorescent stain were 57(14.69%) when compared to Zn stain 29(7.47%).

Comparison between fluorescent staining and genexpert in previous study had showed higher positivity rate which was 63.9% by Fluorescent staining methods and 74.7% were detected by GeneXper method. The results of present study by fluorescent staining which completely concordant with geneXpert was 86.6% , while 13.4% showed detection of MTB by GeneXpert only. Although GeneXpert exerted higher detection rate of positivity of MTB but smear microscope is a simple ,economical, less time-consuming technique and cheaper for detection of acid fast bacilli (AFB) in sputum. Regarding to detection of rifampacin resistant, 2 out nine(22.22%) specimens which MTB detected only by GeneXpert showed rifampcin resistant. 6.4% only showed AFB by fluresent stain and not detected by GeneXpert , so it was consider as Mycobateria other than tuberculosis.

the sensitivity and specificity of ZN staining was 74.6% and 98.8% respectively when compared with geneXpert The present study revealed high**ly** sensitivity and specificity of geneXpert which was 96.2% and 96.1% respectively when compared with ZN staining results, other study by munir et al 2015 showed sensitivity and specificity 90.1 % and 98.3% and these finding were approximately agreement with current study which reflect the curtail need of geneXpert which exert higher advantage of this device in determination of the causative agent accurately in addition to detect the resistant patterns of rifampcin.

In the current study the sensitivity and specificity of ZN staining was 74.6% and 98.8% respectively when compared with fluorescent(AO) staining which revealed high**ly** sensitivity of fluorescent(AO) staining which was 86.6% while the specificity were approximately similar by both methods (specificity97.7%) when compared with ZN staining results, other finding similar to the current study obtained by Timalsina *et al.(* 2015) showed sensitivity and specificity 60.03% and 98.51% for ZN method; and 83.56% and 94.53% for fluorescent AO method respectively,and these finding were approximately agreement with current study which reflect the efficacy of fluorescence microscopy in the diagnosis of pulmonary tuberculosis.

In the current study 2.07%were rifampacin resistant detected by GeneXpert, 5.1 %(3/58) of the rifampacin resistant bacilli was AFB microscopically while the 1.1% (2/179) rifampacin resistance MTB were not detected microscopically and consider smear negative, so the total the total percentage of patients had rifampacin resistant by GeneXpert was 7.4% (5/67) , in 2012 Sudan reported 7.7% multidrug resistant was obtained among new cases who showed smear positive (ibrahum. 2012) other studies reported similar levels of rifampacin resistance 7.2% by Rasaki et al (2014).

and 8.6% by Olusoji *et al.* (2013),while other studies showed high percentage of rifampacin resistance which were 19%, and 29.87% by Lawson *et al.* (2010) and Ganguly *et al.* (2015) respectively and Idigbe *et al.* (1998) who reported only 2% of resistance to rifampacin in Lagos, Nigeria.

The current study consider four out of sixty two patients (6.5%) which their specimens showed smear positive (AFB) only by fluorescent (A-O) staining as having Mycobacterium other than tuberculosis (MOTT) and could not identified to the species level, although the identification of the species of mycobacteria is a very important issue, though non-tuberculosis species may be less pathogenic than M.TB, but they may cause clinically serious disease especially in immunocompromised patients in addition may be wrongly diagnosed as TB patients and received antituberculous drugs , and may some cases showed no response. Acid-fast stains are non-specific and the reader cannot determine the species of Mycobacteria present in a positive smear. Other study by Ibrahim (2012) in Sudan showed 2.4%, regarding the current study it was obviously increase the number of mycobacterium other than tuberculosis and these may be attributed to HIV status , which the atypical is more dominant among HIV patients and other immunocompromised patients. Study by Maghzob 2010) .who reported 84% of patients with HIV were found infected with MTB while 16% were co-infected with NTM.

TB treatments take too long to cure, are too complicated to administer, and can be toxic. Many people have negative interactions between commonly used antiretroviral and TB treatment. People with TB must take drugs from 6 months to 2 years or longer—or risk developing more difficult to treat drug-resistant TB, treatment

for drug-resistant TB can take up to two years, and is so complex, expensive, and toxic that many patients are unable to access treatment. Further, the cost of curing MDR-TB can be staggering — literally thousands of times as expensive as that of regular treatment in some regions — posing a significant challenge to governments, health systems, and other payers. Of those who do, almost half will die.

Direct costs of treatment averaged $134,000 per MDR TB patient and $430,000 per XDR TB patient. In comparison, costs are estimated at $17,000 per non-MDR TB patient.(CDC 2014) In comparison the cost of the GeneXpert device around USD 17,500 per 4-module instrument is significantly higher than for microscopy (around USD1,500 per microscope (WHO 2010) but it is clearly obvious the early detection of rifampacin resistant and enrollment in appropriate treatment regimen will cut the transmission of MDR-TB and subsequently reduce the cost of treatment.

VI. Conclusion

The study concluded that as compared to AFB microscopy, Gene Xpert is more sensitive and specific not only for acid fast bacilli (AFB) detection but also for rifampacin (RIF) resistance. It also helps to avoid injudicious use of anti tuberculosis drugs.

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