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COMPARATIVE STUDY ON SPECIFIC AND EARLY DETECTION OF PULMONARY MYCOBACTERIA COMPLEX USING SMEAR AND CULTURE METHOD AND SEROLOGICAL PATHOZYME EIA KITS

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The objective of this study was to compare the sensitivity and specificity of smear and culture methods with rapid serological EIA myco kits manufactured by Omega diagnostics, for the early detection of *Mycobacterium tuberculosis* (MTB) complex. Sera from various categories of smear and culture results were compared with the result of 38KDa, 16KDa and purified protein for IgA, IgM and IgG antibodies with sensitivity of 4%, 24% and 76%, respectively and with specificity of100% for IgG in Smear and Culture Positive (S^+C^+) category. The sensitivity of the test improved to a level of 80% for IgG + IgA without affecting the specificity. A combination of IgG + IgA and IgM further improved the sensitivity to 88% but reduced the specificity to 91%. Amongst the S^-C^+ and S^-C^- 64% and 14.7% were positive for IgG respectively. The predictive value of the kit using S^+C^+ subject was 96%. For all culture positivity (n=78), there was 2.6%, 33.3% and 71.8% sensitivity for IgA, IgM and IgG respectively. IgA +IgG and IgA + IgM + IgG combination gave 74.4% and 84.6% sensitivity respectively with the same level of specificity. Fifty-five percent of culture positive subjects were found to be MTB complex positive by routine biochemical tests, while 40% through PATHOZYME TB COMPLEX PLUS kit (high positive (H^+) values). When high positivity is combined with low positivity of the same kit ($H^+ + L^+$), 65% of the isolates were found to be MTB complex. Our study showed 88% sensitivity and 91% specificity for combined IgA + IgM + IgG antibodies recorded for MTB (S^+C^+ group) and 85% sensitivity and 91% specificity for all culture positives. Our study has demonstrated that the myco kits and TB complex plus kit produced by Omega Diagnostics are a good tool for specific, early and rapid identification of active tuberculosis for both diagnostic and epidemiological purposes.

Key words: Tuberculosis, diagnosis, comparative, specificity, sensitivity, culture and serological technique.

INTRODUCTION

Globally, pulmonary tuberculosis (TB) is the leading infectious cause of death, killing 3 million people every year (1). An increase in both pulmonary and extra pulmonary TB is taking place in both developed and developing areas of the world, complicated by AIDS pandemic and multi drug resistant species (2). In Nigeria, the tuberculosis situation has worsened in the past few years (3) and more than 80% of all cases of tuberculosis in Nigeria are of the pulmonary form (4). From the public health point of view, there is a definite need for a specific, sensitive and simple means of performing cost-effective and rapid diagnostic tests for pulmonary tuberculosis.

The existing specific and highly sensitive methods of diagnosis lack rapidity. For example, the culture method that takes between 4-8 weeks for a meaningful result to emerge. The radiological method is rapid but lacks specificity and the PCR method is specific and sensitive but rather expensive for routine diagnosis. Again, earlier attempts to develop serodiagnostic tests for pulmonary and extra-pulmonary tuberculosis with crude *Mycobacterium tuberculosis* antigens such as whole culture filtrate, purified protein derivative (PPD) and sonicates in ELISA had limitations. They either lacked adequate sensitivity or specificity or both. Therefore, a search for the identification of a highly specific

and rapid serological method for diagnosis of this ancient killer of man becomes imperative.

Omega's PATHOZYME TB EIAS utilize the immunodominant and highly specific 38KDa antigen. In addition, PATHOZYME MYCO utilizes a highly immunogenic lipopolysaccharide antigen (LPS). The MYCO range includes 3 individual kits for detection of IgG, IgA and IgM antibodies produced in response to infection by bacteria belonging to the genus mycobacterium. PATHOZYME TB COMPLEX plus contains microplates, which are coated with the 38KDa and another highly specific 16KDa antigen. The assay detects IgG antibodies specific to *Mycobacterium tuberculosis* complex namely; *M. tuberculosis, M. africanum* and *M. bovis,* which are largely implicated in bronchopulmonary tuberculosis in Nigeria and the world at large.

The goal of this study was to conduct a comparative analysis of diagnosis of *Mycobacterium tuberculosis* complex using conventional smear and culture method and rapid serological PATHOZYME EIA kit method manufactured by OMEGA Diagnostics, which are believed to be reproducible and specific.

MATERIALS AND METHOD

Study center/population:

The study was carried out in Lagos, Nigeria. One hundred and sixty two samples were collected from new subjects (15 years and above) presenting with bronchopulmonary disorders at Yaba Chest Clinics and Central Public Health Laboratory, Yaba, Lagos. Twenty-two (15 years and above), previously BCG vaccinated normal healthy individuals (NHI) serve as the control group. The study was carried out between October 2000 and August 2001. The samples were processed in the TB reference laboratory of Nigerian Institute of Medical Research Yaba, Lagos, Nigeria. The 162 patients were pre-counseled and their informed consent obtained and a repeated early morning sputa and single venous blood sample were obtained from each subject.

Microscopy/Culture

The sputum samples were decontaminated and concentrated by the modified Petroff method (5), and screened for the presence of acid-fast bacilli (AFB) by both ZiehlNeelsen (ZN) smear microscopy technique and by culture on 2 Lowenstein Jensen slopes. Growth was confirmed by culture smear demonstrating AFB. Four biochemical analyses were conducted on the isolates.

Serological procedure

The venous blood was put in clean new non-anticoagulated bottles. The blood was left to clot for one hour, the serum removed and subsequently stored at – 4°C until processed. The Serological procedures were conducted according to the manufacturer's technique. Both the reagents and the sera were brought to room temperature and 1/100 dilution of the sera used. The optical density (OD) were read at 450 nm and blanked on air with basic EIA reader (Genscreen Bio-Tek instruments).

Calculation and interpretation of results

Assay validations were shown to comply with the manufacturers standard. The OD of IgM low positive control, and IgG, IgA 2u/ml controls were > 0.20. (Manufacturer's standard). The control sera were each plotted on the semilog graph sheet provided and various computations and extrapolations were done according to the manufacturers protocol guide as follows;

Sero-units:	IgG	IgA
Negative Results	<400u/ml	>300u/ml
Low positive	400u/ml- 900u/ml	300u/ml-600u/ml
Positive Result	>900u/ml	>600u/ml

For IgM, cut off is OD of the average low positive control (LPC); Negative result is OD less than the OD of the low positive control, Low positive result is OD between the of LPC and (OD of the LPC X 1.5), and Positive result is an OD greater than the OD of LPC X 1.5.

RESULTS

Sputum and serum samples were collected from a total of 184 subjects, 102 (55.4%) male and 82 (44.6%) females, aged 15 years and above. One hundred and sixty two (88%) were patients presented at the Yaba Chest Clinic and Central Public Health Laboratory Yaba, with bronchopulmonary infections and 22 (12%) were previously BCG vaccinated normal healthy individuals.

Table 1 shows the serum antibody levels of isotypes IgG, IgA and IgM and TB complex plus to the purified antigen determined in subjects with new active smear and culture positive (S^+C^+) (n=50), smear positive and culture negative (S^+C^-) (n=16), smear negative and culture positive (S^-C^+) (n=28), smear negative and culture negative (S^-C^-) (n=68) subjects and normal healthy individuals (n=22).

Table 2 shows the positivity rate in the polar groups S^+C^+ and NHI. Thirty-eight out of 50 patients were positive for IgG antibodies giving a sensitivity of 76%. All the 22 NHI control sera were negative for IgG antibodies making TB IgG 100% specific. The positivity rates for other classes were low, for IgA (4%) and IgM (24%) antibodies, although there was specificity of 100% and 91% respectively. It was further observed that some of the sera, which were negative for IgG antibodies, were positive for IgA and/or IgM antibodies. When we combine the sera positive for IgA in those positive for IgG, 2 additional sera were included with none positive from the healthy normal individuals, maintaining a specificity of 100%, but very low sensitivity of 4% for IgA. Additional 4 sera were equally positive for IgM alone, when combined with those also positive for IgM in the IgG positive group, 12 patients became positive with a sensitivity of only 24%. Two normal healthy individuals were positive for IgM, reducing the specificity to 91%.

When we combine positivity for IgG and IgA (IgG + IgA), an increase in sensitivity to 80% (40/50) was recorded with 100% specificity. A combination of IgG + IgA +IgM, gave 4 additional positive results increasing the overall sensitivity of the test to 88% but reducing the specificity to 91%.

The overall predictive value of the positive results for IgG +IgA + IgM for the category S^+C^+ was 96%. It was observed that when the High positive (H^+) and Low positive (L^+) were combined for S^+C^+ sera, sensitivity of 100%, 36% and 60% were obtained for IgG, IgA and IgM respectively (Table 1). Amongst the S^+C^- (n=16), no High positivity was recorded for IgG, but 6 (37.5%) had Low positivity, while in S^-C^+ group (n=28), 18 (64.3%) had H^+ and 10 (35.7%) L^+ results. For S^-C^- clinical TB bronchopulmonary disease subjects (n=68), 10 (14.7%) had H^+ and 8 (11.8%) had L^+ results for IgG.

Table 3 demonstrates the positivity of all culture positive subjects (S^+C^+ + S^-C^+) (n=78). It showed that 66 (84.6%) were detectable serologically using the kits with 71.8% IgG sensitivity. For TB complex plus, which utilizes 38KDa and 16KDa antibodies specific for MTB complex, 22(28.2%) were H^+

from all culture positive subjects ($S^+C^+ + S^-C^+$), while 36 (46.2%) were positive when H^+ and L^+ were combined. This showed that 22 of 78 by high positivity (H^+) and 36 of 78 by high positivity plus low positivity ($H^+ + L^+$) were *M. tuberculosis, M. bovis or M. africanum.*

From culture morphology, period of growth, pigmentation and biochemical analysis, 55% of the isolates studied were found to be MTB complex.

TABLE 1: EIA positivity is sera of the subjects; smear and culture categories

Isotype:		**Smear and Culture category:**				**NHI**
		S^+C^+ [n=50] n(%)	**S^+C^- [n=16] n(%)**	**S^-C^+ [n=28] n(%)**	**S^-C^- [n=68] n(%)**	**[n=22] n(%)**
	H^+	38(76)	0(0)	18(64.3)	10(14.7)	0(0)
	L^+	12(24)	6(37.5)	10(35.7)	8(11.8)	0(0)
IgG	$L^+ + H^+$	50(100)	6(37.5)	28(100)	18(26.5)	0(0)
	N	0(0)	10(62.5)	0(0)	50(73.5)	22(100)
	H^+	2(4)	2(12.5)	0(0)	4(5.9)	0(0)
	L^+	16(32)	0(0)	2(7.1)	6(8.8)	0(0)
IgA	$L^+ + H^+$	18(36)	2(12.5)	2(7.1)	10(14.7)	0(0)
	N	32(64)	14(87.5)	26(92.9)	58(85.3)	22(100)
	H^+	19(36)	4(25)	6(21.4)	12(17.6)	2(9.1)
	L^+	12(24)	0(0)	12(42.9)	6(8.9)	2(9.1)
IgM	$L^+ + H^+$	30(60)	4(25)	18(64.3)	18(26.5)	4(18.5)
	N	20(40)	12(75)	10(35.7)	50(73.5)	18(81.8)
	H^+	14(28)	0(0)	8(28.6)	4(5.9)	0(0)
TB COMPLEX	L^+	8(16)	4(25)	6(21.4)	12(17.5)	0(0)
plus	$L^+ + H^+$	22(44)	4(25)	14(50)	16(23.5)	0(0)
	N	28(56)	12(75)	14(50)	52(76.5)	22(100)

Key

S^+C^+ = Smear and culture positive; S^+C^- =Smear positive and culture negative

S^-C^+ = Smear negative and culture positive; S^-C^- = Smear and culture negative

NHI = Normal Healthy individuals; H^+ = High positive; L^+= Low Positive; N = Negative

TABLE 2: Kits Positivity in the Polar Groups; High Positive Group for S^+C^+

Isotype	S^+C^+ [n=50] n^+	%SEN	NHI [n=22] n^+	%SP
IgG	38	76	0	100
IgA	2	4	0	100
IgM	12	24	2	90.9
IgG + IgA	40	80	0	100
IgG+IgA+IgM	44	88	2	90.9

KEY: S^+C^+= Smear and culture positive; SEN = Sensitivity; SP = Specificity; n^+ = Number positive

Assay Validation: IgA and IgG 2U/ml control OD, must be > 0.2. Result: IgA = .239, IgG = .317

For IgM low positive control OD, must be > 0.2. Result; = .283

TABLE 3:

Positivity in the Polar Group Positive S^+C^+ and S^-C^+ (all culture positive groups)

ISOTYPE	ALL POSITIVE GROUP (S^+C^+ and S^-C^+) (n=78)	%SEN	%SP
IgG	56	71.8	100
IgA	2	2.6	100
IgM	26	33.3	90.9
IgG + IgA	58	74.4	100
IgG+IgA+IgM	66	84.6	90.9

DISCUSSION

The lack of adequate sensitive and specific early diagnosis of MTB has been one of the major problems in containing the spread of the disease. Existing methods of TB identification have various shortcomings; Acid Fast-Bacilli (AFB) smear microscopy, requires the highest number of AFB in the sputum, requires at least 3 visits to a health facility by the patient and is labour intensive, time consuming and misdiagnosis is apparent. It has 50% sensitivity, but may be as low as 30%, and 98% specificity (2). Infact, World Health Organization in 1998 declared that bacteriological culture of tuberculosis is notoriously slow, difficult and expensive and the WHO guide concentrates on the exact

procedures and precautions needed to prevent errors and ensure reliable results.

In laboratories, increase pressure of work per day may not enable enough time to properly read each slide, therefore, the sensitivity is reduced to 25-35%. However, the culture method is still the most sensitive and specific for the identification of MTB, although results are delayed for weeks or months. Largely speaking, smear negative subjects are risks to the entire population. The disadvantages of microscopic based MTB diagnosis methods motivated early serological studies by Nassau *et al* in 1976 (6). Daniel and Anderson (7) reported 72% and 94% sensitivity and specificity respectively of species specific antigen 5 of *M. tuberculosis*, which was later proven to be the same as 38KDa antigen (8). The 38KDa antigen isolated by different methods has been used in diagnostic tests (mainly ELISA) with varying outcomes. As antigen 5 by Nassau *et al*, showed high specificity in all the studies conducted in the world (88-98%), while sensitivity ranged from 49% to 89% (8, 9,10,11, 12).

The 38KDa antigen used in the Omega Myco and TB plates was similar to those reported, in its ability to detect smear positive cases. In all the cited studies, antibody of class IgG alone had been measured except for study by UmaDevi *et al* in 2001 (8), which added IgA and IgM. This was after Omega diagnostics had produced their Myco kits. The scientific reason behind the combination of these antibodies was the fact that some sera that were negative for IgG were still positive for IgA and IgM antibodies. Generally, IgM detects more recent infection and this was indicated on the high level of IgM antibody detected from S^-C^+ subjects (n=28), but the significance of the use of IgA and IgM is questionable since Lagos, Nigeria is an endemic area for tuberculosis and many adults used for the study may have been exposed and infected with the bacilli.

The differences between our results and lower sensitivity reported by some others may be explained by the mode of preparation of the antigen reference. For instance, Omega used a recombinant 38KDa protein and a highly purified antigen derived from *Myco bacterium tuberculosis*, while UmaDevi *et al* used a 2-D preparative electrophoresis for the purification of their 38KDa antigens. The addition of 16KDa antigen for TB complex plus made the kit species specific. For Myco complex, sensitivity was shown for smear-positive and culture negative (S^+C^-) cases (n=16) and also with S^-C^- subjects, which had no way of correlating with the existing standard i.e. culture positively, otherwise, the high specificity and sensitivity recorded by these kits would have been a panacea to all important early and specific diagnosis of MTB.

From the result, 76% all S^+C^- subjects were of high positivity category, the remaining 24% were of low positivity. Since none of the S^+C^+ subjects were negative, we suggest that cases of low positivity, which presents with all symptoms of bronchopulmonary tuberculosis be regarded as positive for MTB. We therefore recommend a further addition of kits for measuring circulating immune complex (CIC) of anti-38KDa antibodies which would greatly improve the sensitivity and specificity of the kits and may help to buttress results acceptance for S^-C^+ cases as reported by UmaDevi *et al* (8), who reported that 45 of 55 S^-C^+ subjects were positive for CIC antibodies plus serum IgG, while 44 of 64 S^-C^- were

positive. An addition of a kit to measure anti-38KDa circulatory immune antibodies will equally help reduce false positives and hence improve sensitivity and specificity.

Our study has demonstrated that the Myco kits and TB complex plus kit produced by Omega Diagnostics are a good tool for specific early and rapid identification of active tuberculosis for both diagnostic and epidemiological purposes.

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