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IL-10, IL-17 and VEGF as Biomarkers for Differentiating Tuberculosis in North-Western Nigeria

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IL-10, IL-17 and VEGF as Biomarkers for Differentiating Tuberculosis in North-Western Nigeria

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Abstract

Background: Cytokines mediate resistance to tuberculosis infection and play a significant role in host susceptibility and the progression of the infection. Aim: This study aimed to determine whether plasma levels of IL-17, IL-10, and VEGF can discriminate between non-resistant TB, monoresistant TB, Multidrug-resistant TB, and TB-negative subjects. Methods: Three hundred and twenty-five presumptive TB patients were recruited from 2020 to 2023 in the North-western region of Nigeria as study participants from whom sputum and blood samples were collected. The sputum samples were processed accordingly to detect drug-susceptible, monoresistant, and multidrug-resistant Mycobacterium tuberculosis (MDR-TB) using the Ziehl-Neelsen staining technique, GeneXpert, and Lowenstein-Jensen medium, based on which the participants were categorised into three groups: the drug-susceptible group, monoresistant group, and MDR-TB group. In contrast, the blood samples were used to determine the IL-10, IL-17 and VEGF plasma levels of all the groups using the Melsin ELISA test kit. Results: In all three groups, the plasma level of VEGF was the most elevated; however, there was a significant statistical association between IL-17, and VEGF when the groups were compared (p = 0.000). The Receiver operating characteristic curve (ROC) assessment for IL-10, IL-17, and VEGF showed that the area under the curve (AUC) for IL-17 was the highest for all the three groups (0.743, 0.858, and 0.99). Conclusion: This study indicates IL-17 as the best overall diagnostic performance as a discriminator between non-resistant TB, Mono-resistant TB and the MDR-TB group.

Keywords: IL-10, IL-17, VEGF, Tuberculosis

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) and is transmitted through aerosol droplets released when an infected person coughs.[1] Despite a slowdown in the annual increase in TB cases and a decline in TB-related deaths, the disease remains a significant public health threat with 10.8 million new cases reported in 2023.[2]

Cytokines play a significant role in host susceptibility and progression of TB.[3] Meanwhile, IL-10 is a cytokine that can be produced by myeloid cells and was also reported to have a certain correlation with active and latent TB.[4–6] Research that has been conducted shows that IL-10, which has an important role in regulating the immune response, can also influence the development of TB.[7] IL-17 cytokine is a proinflammatory cytokine that primarily mediates resistance to extracellular bacteria and fungi but is also associated with host immunity to TB.[8] A subpopulation of granuloma macrophages produces Vascular Endothelial Growth Factor-A (VEGF-A), which recruits immune cells to the granuloma by a non-angiogenic pathway.[9]

The hallmark lesion of TB pathology is the tuberculous granuloma, a compact mass of immune cells comprised of lymphocytes, macrophages, epithelioid cells, Langerhans cells, fibroblasts, DCs, and natural killer cells (NK).[10]

Th17 cells play a crucial role in immunity against *M. tuberculosis*, as reduced Th17 responses were associated with the severe outcome of *M. tuberculosis* infection.[11]

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VEGF is a tyrosine kinase glycoprotein that plays a vital role as a mediator of angiogenesis and vasculogenesis in both physiological and pathological processes, implicated in numerous inflammatory and chronic conditions.[12] and can be utilised as a promising biomarker to distinguish active tuberculosis from latent TB.[13]

2. Methods

2.1. Study Area

The study areas covered are Kano, Katsina, Jigawa, Kaduna and Zamfara States of Northwestern Nigeria. Six hospitals within the aforementioned States were used as collection centers: Aminu Kano Teaching Hospital (AKTH), and Infectious Diseases Hospital, Kano State; Federal Medical Centre, Katsina; Federal Medical Centre, Birnin Kudu, Jigawa State; National Tuberculosis and Leprosy Training Centre (NTBLTC) Saye, Zaria, Kaduna State; and Federal Medical Centre, Gusau, Zamfara State.

2.2. Study Design

The study is a cross-sectional study in which prevalence of non- resistant *M. tuberculosis* and MDR-*M. tuberculosis* infections were determined in presumptive TB patients and associated risk factors in relation to cytokine response and single nucleotide polymorphisms in IL-10, IL-17 and VEGF.

2.3. Study Population

The study is a cross-sectional study in which SNPs in IL-10, IL-17 and VEGF were determined in relation to MDR-TB. Study participants included presumptive tuberculosis patients attending the aforementioned selected hospitals in North-western Nigeria.

2.4. Inclusion Criteria

HIV negative presumptive tuberculosis patients within the range of age18–60 years, attending TB Clinic at the aforementioned selected hospitals. Only those who consented at the time of sampling were included in the study.

2.5. Exclusion Criteria

(i) HIV reactive TB patients (ii) Patients not within the age range of 18–60 years.

2.6. Sample Size Determination

The sample size was calculated as follows, using the formula of Sarmukaddam and Gerad,[14] and a prevalence of 25.5% obtained in Nigeria:[15]

$$n = \frac{Z^2 Pq}{d^2}$$

Where n = number of sample
z = standard normal distribution at 95% confidence limit = 1.96
p = prevalence of previous study 25.5% = 0.255.[15]
q = (1 – p) = 1 – 0.255 = 0.745
d = allowable error, which is taken as 5% = 0.05

$$\frac{(1.96)^2 \times 0.255 \times 0.745}{(0.05)^2} = 292$$

A sample drop out of about 10% is expected, and to deal with this an increase in attrition rate of 10% was decided on the sample size = 29
Therefore, sample size = 29 + 292 = 321 (rounded up to 325).
Thus, total of 325 sputum and blood specimens were collected from presumptive TB patients for the study. A structured questionnaire was used to obtain bio data and demographic features of the patients; and risk factors among the patients.
The 325 samples were shared among the five study site States based on population ratio of each state as follows:

1. Total population of the five states was calculated = 9,401,288 (Kano) + 6,113,503 (Kaduna) + 5,801,584 (Katsina) + 4,361,002 (Jigawa) + 3,278,873 (Zamfara) = 28,956,250.[16]
2. The ratio of the sample size for each State was determined by calculating the proportion of the population that each State represents by dividing the population of each State by the total population (Table 2.1).

Thus, 37 samples were collected from Federal Medical Centre Gusau; 49 from Federal Medical Centre Birnin Kudu; 65 from Federal Medical Centre; 69 from National Tuberculosis and Leprosy Training Centre and Referral Hospital Saye, Zaria-Kaduna State and 105 from both AKTH and Infectious Diseases Hospital (IDH) (that is, 61 from IDH and 44 from AKTH (Table 2.1).

2.7. Collection and transport of samples

Sputum samples (5 ml) was collected from patients suspected to be infected with *M. tuberculosis*

Table 2.1. Sample size for each state.

State	Population(n)	Population ratio(z) = n/N (where N = 28,956,250)	Sample size(S) = zN
Kano	9,401,288	0.32	105
Kaduna	6,113,503	0.21	69
Katsina	5,801,584	0.20	65
Jigawa	4,361,002	0.15	49
Zamfara	3,278,873	0.11	37
Total	28,956,250		325

attending the aforementioned selected Hospitals in Northwestern Nigeria using sterile wide mouth (50 ml) falcon tube and labeled appropriately. Specimens were stored at 2–8 °C at sample collection sites until transported to AKTH TB laboratory using cold chain.

Four millilitres of blood samples were collected from same patients into a sterile EDTA container using vacutainer needle and stored at 2–8 °C.

3. Microbiological analysis

3.1. Ziehl-neelsen stain

3.1.1. Staining Procedure

The fixed smears was flooded with a solution of 1% basic fuchsin. The smear was gently heated until steaming with a Bunsen burner for five minutes. The smear was then rinsed with water and decolourized with 1% acid-alcohol. It was allowed to stand for two minutes. The smear was then rinsed with water and counterstained with methylene blue for two minutes. The slide was finally rinsed with water and air dried before examination. The stained smears were scanned with ×100 oil immersion lens for the presence of red thin rods or coccobacilli in accordance with Kurup and Chester (2014) method.[17]

3.2. Molecular identification assay

3.2.1. Assay procedure

The procedure according to Lawn & Nicol,[18] was used for the assay. The assay utilizes single-use plastic cartridges with multiple chambers that are preloaded with liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction and heminested rt-PCR. Clinical sputum samples (or decontaminated sputum pellets) were treated with a sodium hydroxide and isopropanol-containing sample reagent (SR).The sputum container was carefully unscrewed, The SR was then added to the sample at a 2:1 and was shaken vigorously for minutes after replacing the lid. It was then incubated at room temperature for 15 min. It was further shaken for 15 minutes and then incubated again for 5 minutes. This step aimed at reducing the viability of *M. tuberculosis* in sputum at least 10^6-fold to reduce biohazard risk. It was ensured that sample became perfectly fluid before being tested. Samples that remained viscous were kept for 10 further minutes before inoculation into the cartridge. The treated sample was then manually transferred to the cartridge which was then loaded into the Gene Xpert instrument. Subsequent processing was fully automated.

3.2.2. Phenotypic drug susceptibility test (DST)

The Sputum samples were decontaminated with N-acetyl-L-cysteine sodium hydroxide in accordance with the Central TB Division[19] guidelines for *M. tuberculosis* Culture & Drug susceptibility testing by proportion method. After centrifugation at a speed of 3,000 for 15 min, the pellet was suspended in 1 ml phosphate-buffered saline. It was inoculated on two Lowenstein-Jensen (L-J) media slants which were incubated at 37 °C for 8 weeks depending on the time required for the organisms to become evident. Mycobacterial growth was examined on weekly basis. The *M. tuberculosis* isolates were identified according to growth rate and colony morphology.

Phenotypic DST was carried out on confirmed *M. tuberculosis* isolates in accordance with World Health Organization Technical Manual for Drug Susceptibility Testing of Medicines used in the treatment of TB.[20] The test was performed on L-J media containing Isoniazid (0.2 μg/ml), Rifampicin (40 μg/ml) and Ethambutol (2 μg/ml). Inocula were cultured in a 37 °C incubator for 8 weeks, and the results were interpreted as susceptible or resistant accordingly. The standard criterion of the proportion method for classifying a strain as resistant was the ratio of the number of colonies obtained on drug-containing medium to the number of colonies obtained on drug-free medium (growth of $\geq$ 1% of colonies). Drug resistance was defined as any resistance to Rifampicin and any other one or more first-line drugs. Monoresistance was defined as resistance to only one of the 3 drugs.[21]

4. Immunological analysis

4.1. IL-10, IL-17 and VEGF assay

4.1.1. Assay procedure

All samples were tested for anti-IL-10, anti-IL-17 and anti-VEGF using the IL-10, IL-17 and VEGF ELISA test kits purchased from Melsin Medical Co., Ltd, Changchun, China with adaptation of the procedure of Lequin (2005).[22] Dilutions of standard was prepared to get a concentration of 240ng/l, 160ng/l, 80ng/l, 40ng/land 20ng/l. 50μl of standards were pipette into the standard wells. Forty microliter (40μl) of sample diluents was dispensed into each sample well after which 10μl of sample was dispensed into each of the wells. Chromogen solution A and B, and stop solution were added into the blank well. Fifty microlitre (50μl) of HRP-conjugate reagent was added to all wells except blank. The plates were then covered with an adhesive strip and incubated for 30 minutes at 37 °C. The plates were washed 4 times. Fifty microlitre (50μl) each of chromogen solutions A and B were added to each well. They were incubated for 10 minutes at 37 °C. Fifty microliter (50μl) of stop solution was added to each well. The optical density of the samples was read immediately in microtititre plate reader at 450 nm wavelength within 15 minutes.

4.2. Statistical analysis

The pattern and trend of the data obtained was investigated using SPSS software. Data were subjected to normality and Homogeneity of variance tests using the Shapiro-Wilks W test and Levene's Test respectively. *P* value of less than 0.05 for both the Shapiro-Wilks W test and Levene's test was presumed as an indicator of non-normal and non-homogenous data respectively while a non-significant *p* value (< 0.05) was taken as an implication for normal or homogenous data as the case may be. Any data whose values deviated from normal/homogenous equality was analysed using non-parametric tests.

Comparisons using Mann–Whitney U test were made between independent variables (that consist of two categorical independent groups), and continuous dependent variables that were not normally distributed. The two categorical independent variables were: Susceptible TB Vs TB negative; MDR-TB Vs TB negative; Susceptible TB Vs Mono-resistant TB; MDR-TB Vs Monoresistant TB and Monoresistant Vs TB negative; while the dependent continuous variables were: IL-10; IL-17 and VEGF plasma levels. For multiple groups, the Kruskal–Wallis test was used to compare the IL-10; IL-17 and VEGF plasma levels (which appeared not normally distributed) of the 4 independent groups: Susceptible TB; MDR-TB; Monoresistant TB and TB negative.

The accuracy of plasma IL-10, IL-17 and VEGF levels as biomarkers to screen for MDR-TB/TB was summarised in a non-parametric receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) was used as a measure of forecast quality. The discriminant capacity (to figure out MDR-TB/TB) of the IL-10, IL-17 and VEGF plasma levels was considered in accordance with Ozdemir & Algin,[23] that is, If the AUC value is 0.90–1.00, it is excellent, 0.80–0.90 is good, 0.70–0.80 is medium, 0.60–.070 is weak, 0.50–0.60 is unsuccessful.

5. Results

The three cytokines were higher in the MDR-TB participants with VEGF being significantly higher. The result showed a significant statistical difference among IL-10, IL-17 and VEGF across non-resistant TB, mono-resistant TB and MDR-TB patients ($p = 0.000$ for all the cytokines). Comparison between non-resistant TB and participants that were negative to TB showed statistically significant difference for the three cytokines ($p = 0.000$ for IL-10, IL-17 and VEGF respectively). Comparing MDR-TB with Negative TB patients also revealed a statistically significant difference for IL-10 ($p = 0.002$), IL-17 ($p = 0.000$) and VEGF ($p = 0.000$) while non-resistant TB Vs mono-resistant TB showed statistical difference for VEGF only ($p = 0.000$). There was no statistical difference for all the three cytokines in MDR-TB Vs mono-resistant TB patients, however, IL-17 and VEGF were statistically significant in the mono-resistant Vs TB negative patients ($p = 0.001$ and 0.000 for IL-17 and VEGF respectively (Table 5.1).

6. Discussion

This study found that plasma IL10, IL17 and VEGF responses were higher in the MDR-TB patients than in the, mono resistant and non-resistant-TB patients ($p = 0.000$) and in the TB negative patients ($p = 0.003$) with VEGF being significantly higher; perhaps because granuloma macrophages produce VEGF, which recruits immune cells to the granuloma which may have resulted to high levels of VEGF in TB.[7]

The Mann Whitney U test revealed a strong statistical association for IL10, IL17 and VEGF in non-resistant TB Vs TB negative, MDR-TB Vs Negative TB. For non-resistant TB Vs monoresistant group, only VEGF was found to be statistically associated with

Table 5.1. Relationship between IL-10, IL-17 and VEGF levels and TB, MDR-TB and Mono Resistant TB.

Variable	Susceptible TB (n = 42)	MDR-TB (n = 10)	Mono-Resistant TB (n = 5)	Negative (n = 268)	4 group Test	Susceptible TB Vs TB Negative	MDR-TB Vs TB Negative	Susceptiblet TB Vs MonoResistant	MDR Vs Monoresistant	Monoresistant Vs TB Negative
			Kruskal-Wallis						**Mann Whitney *U***	
IL-10	11.50 (5.99)	14.21 (8.10)	13.66 (6.22)	6.96 (3.08)	0.000	0.000	0.002	0.408	0.903	0.008
IL-17	23.00 (13.78)	71.63 (28.12)	51.48 (14.55)	12.73 (4.90)	0.000	0.000	0.000	0.317	0.111	0.001
VEGF	50.22 (49.62)	217.13 (146.88)	149.18 (236.25)	18.58 (25.2)	0.000	0.000	0.000	0.000	0.220	0.000

Key: IL-10 = Interleukin 10, IL-17 = Interleukin 17, VEGF = Vascular Endothelial Growth Factor, TB = tuberculosis, MDR-TB = Multidrug resistant tuberculosis, n = number, Vs = Versus, P-value≥0.05 = statistically non-significant; P-value≤0.05 = statistically significant

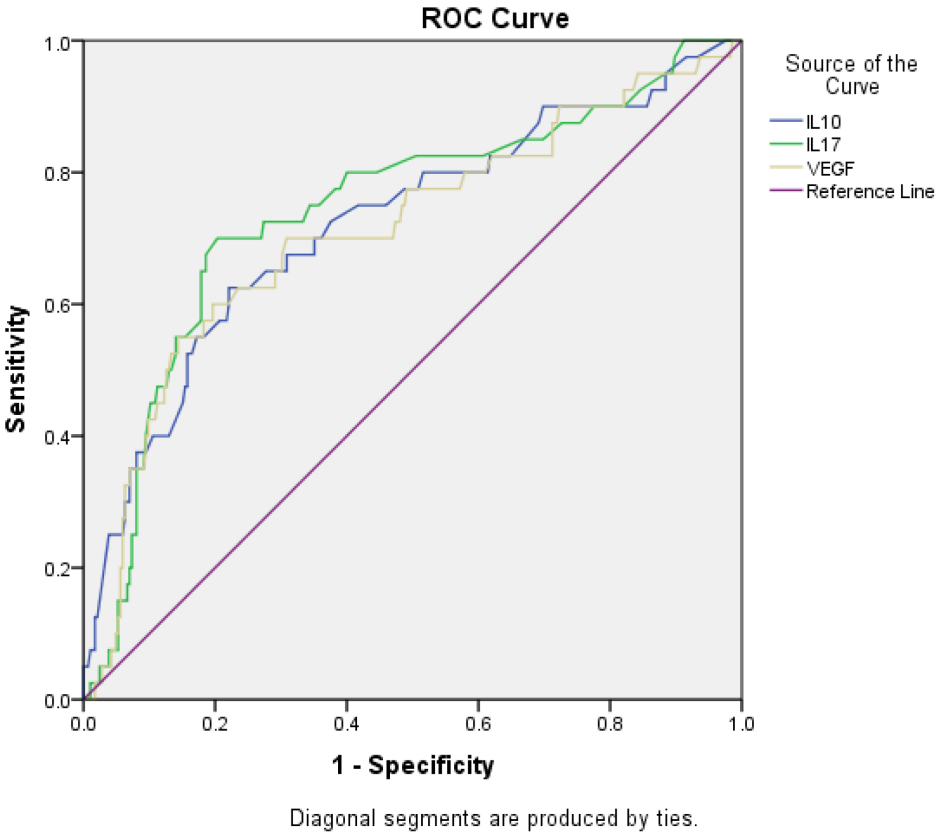

Fig. 1. ROC curve for Non-resistant TB. The AUC for IL-10, IL-17 and VEGF was 0.722, 0.743 and 0.712 respectively. It shows the Receiver Operating Characteristics (ROC) Curve for Non-resistant TB patients (Susceptible TB). IL-17 appears closer to the upper left corner (coordinate [$x = 0, y = 1$]) and thus, more sensitive and more specific as a predictor for susceptible TB than both IL-10 and VEGF. The Area under the Curve (AUC) for IL-17 is 0.743, which is moderately good for a suitable biomarker; higher than 0.722 and 0.7 for both IL-10 and VEGF respectively; however both IL-10 and VEGF have the capacity of a fairly good discriminator of non-resistant TB as well although VEGF appears more less sensitive and less specific by virtue of being closer to the diagonal (line of equality).

the group ($p = 0.000$), however, there was no statistical association in all the cytokines for the MDR Vs Monoresistant group as opposed to the monoresistant Vs TB negative group where all the three cytokines were statistically associated with the group. VEGF showed quality of a good discriminator of MDR-TB as shown in the findings.

The study suggests that all the 3 cytokines evaluated differentiate between TB-infected (whether MDR, monoresistant or non-resistant) and TB-uninfected individuals. A Kruskal-Wallis test revealed that there was a significant effect of MDR-TB on VEGF level (217pg/ml) when compared to negative TB (18.58pg/ml), monoresistant TB (149.18pg/ml) and non-resistant TB (50.22pg/ml). This significant elevation of plasma VEGF level in MDR-TB applies to IL10 and IL17. The ability of *M. tuberculosis* to cause infection lies on its capacity to thwart the innate immune response. For Non-resistant TB, IL17 has greater AUC than IL10 and VEGF, although all of them are fairly diagnostic The Area Under the curve (AUC) for IL-10, IL-17 and VEGF was 0.722, 0.743 and 0.712 respectively. The AUC for IL10, IL17 and VEGF was 0.759, 0.858 and 0.811 respectively for IL10, IL17 and VEGF for Mono-resistant TB group. IL17, a proinflammatory cytokine, appeared more sensitive and more specific than IL10 and VEGF in monoresistant TB patients and hence its association with host immunity to TB is clear. The Area Under the curve (AUC) for IL10, IL17 and VEGF in MDR-TB patients was 0.752, 0.990 and 0.974 respectively. It indicates VEGF as the highest discriminator between non-resistant TB, Mono-resistant TB and the MDR-TB group; also, VEGF has the highest capacity for distinguishing between the 3 groups of TB patients which is similar to a laboratorial study conducted in health units located in Rio de Janeiro state, Brazil that showed VEGF presented the highest AUC value of 0.89.[24]

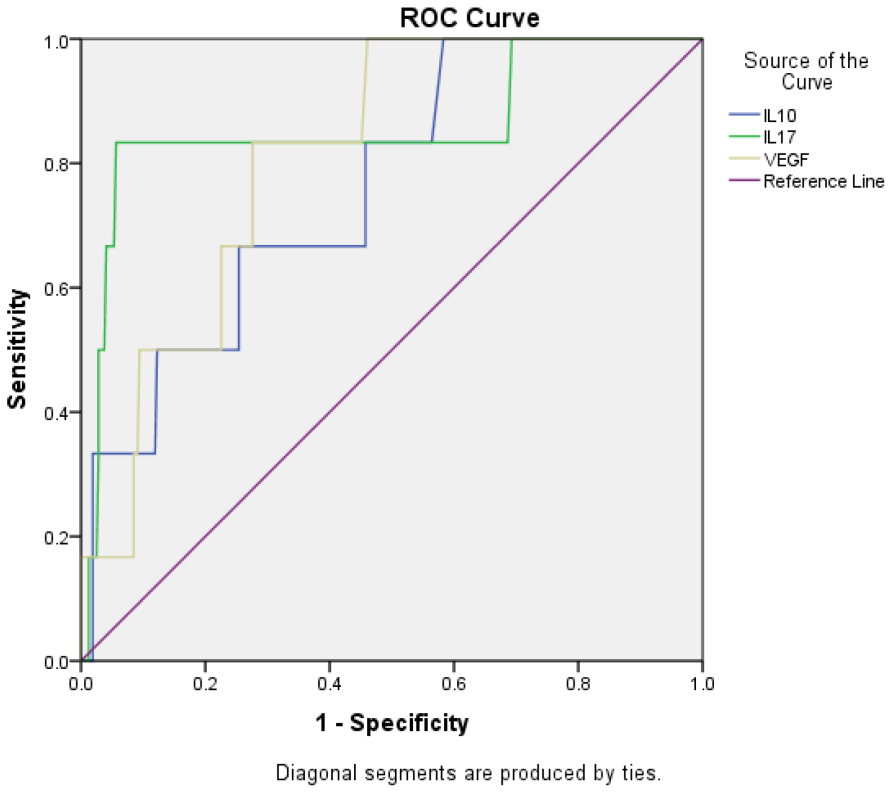

Fig. 2. ROC curve for IL10, IL17 and VEGF for Mono-resistant TB group. The Area Under the curve (AUC) for IL10, IL17 and VEGF were 0.759, 0.858 and 0.811 respectively.

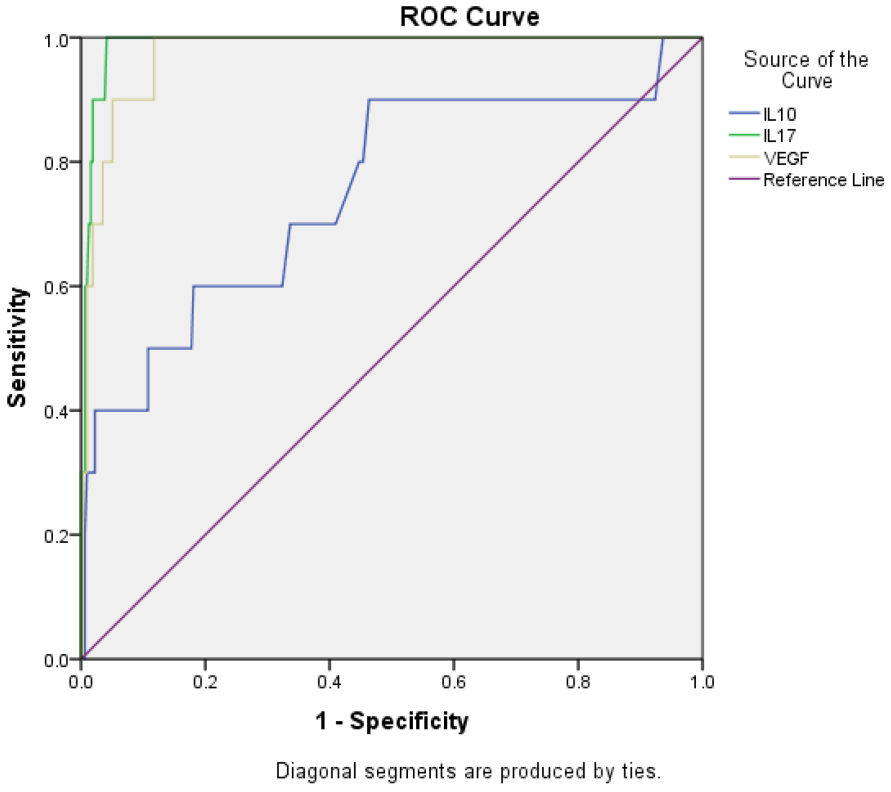

Fig. 3. ROC for IL10, IL17 and VEGF for MDR TB group. The Area Under the curve (AUC) for IL10, IL17 and VEGF was 0.752, 0.990 and 0.974 respectively.

Ethical approval

Confidentiality was assured by using code and permission obtained from the Ethical Committees of Aminu Kano Teaching Hospital (Ref.Number: AKTH/EC/2449AKTH/MAC/SUB/12A/PG/VI/2519); Kano State Ministry of Health (Ref. Number: MOH/797/T.I/1124); Federal Medical Centre Birnin Kudu (Ref. Number: FMC/HREC/APP/CLN/001/1/185); Jigawa State Ministry of Health (Ref. Number: MOH/SEC.3/S769/I); Federal Medical Centre Katsina (Ref. Number:FMCNHREC.REG.N003/082012); National Tuberculosis and Leprosy Training Centre and Referral Hospital Saye, Zaria-Kaduna State (Ref. Number: NTBL/TRC/ZA/182/Vol.IV) and Federal Medical Centre, Gusau Zamfara State.

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Conflicts of interest

None

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Author contribution

The author conceptualized and designed the research protocol, wrote and edited the manuscript, developed the proposal, and sought ethical approvals from concerned IRBs and did the experimentation.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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