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INVESTIGATING SELECTED *MYCOBACTERIUM TUBERCULOSIS* (MTB) GENES IN FOUR AREAS OF CROSS RIVER STATE.

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Abstract

Mycobacterium tuberculosis (MTB) is highly implicated in human tuberculosis. This infection remains the major public health concern with increase mortality and morbidity in children and adults worldwide. The study of the different MTB strains infecting humans within a given locality is not fully explore in Cross River State (CRS). Therefore, the present study aimed at investigating the MTB strains common in Yakurr, Ugep, Calabar South and Calabar Municipal in CRS using Standard Molecular Techniques. A total of 50 sputum samples were screened for MTB positive for GeneXpert, Five that read positive high (MTB-PH) out of the 50 were randomly selected from each LGA making a total of 20 for a rescreened using BACTEC MGIT 960 broth culture method. DNA isolation, sequencing and blast were carried out on BACTEC MGIT positive cases. 1500bp and 1000bp ladder was a standard DNA ladder used to size samples for 16s rDNA and 500bp used for rpoB genes respectively. Gel electrophoresis for 15 samples from Ugep, Calabar South and Calabar Municipal for 16s rDNA and rpoB were about 1500bp and 400bp accordingly. The result of the blast alignment of the nucleotide sequence reveals that MTB strains (**16s rDNA and rpoB)** were 90-100% closely related and similar to strains currently infecting people in the United Kingdom and FARGO while rpoB were also identified to be such common in Belarus, Minsk, and FARGO as confirmed by the phylogenetic trees. The study is providing a novel information about MTB strains covering four areas in CRS. It is a call for more research in other LGAs in and outside the state for documentation of more scientific data that will provide a useful information for epidemiological survey, non-governmental organizations (NGOs) as well as pharmaceutical industries in designing and developing new lines of effective and more potent drugs that will be able to combat resistance strains and curb the spread of this infection.

Keywords: MTB (16S rDNA and rpoB, phylogenetictree, *Mycobacterium tuberculosis)*

I. Introduction

Mycobacteria are gram-positive, aerobic bacteria. They are divided into three groups namely: *Mycobacterium tuberculosis* complex (MTC (causative pathogen of tuberculosis), non-tuberculosis mycobacteria (NTM), and mycobacterium leprae (causative pathogen of leprosy) [1]. This organism is very successful in posing serious threat to global health [2, 3]. Infection caused by this pathogen may involve many organs such as the lung, liver, spleen, kidney, brain, and bones.

About 32% of the world population (approximately 1.8 billion persons) are infected with *M. tuberculosis*. Of this number, 254, 000 are found in sub-Saharan Africa [4].

Tuberculosis is a major deadly disease affecting both children and adults especially immunocompromised individuals in developing countries, causing the death of more than 80,000 children annually, with more than 500,000 new

cases arising yearly. Nigeria ranked 13th among countries in the world with TB-burden in 2012 and 3rd in 2014 [5] (WHO 2014). In TB endemic regions, a normal host immune response may be sufficient to contain the infection and prevent clinical manifestations. However, the unmitigated infection may result in increased morbidity and mortality [6].

The success of *M. tuberculosis* as a pathogen is attributed to its extraordinary capacity to adapt to environmental changes throughout its course of infection and its insensitivity to the most known antibiotics. It can precisely sense the host immune responses and adequately adapt to their lifecycle. The bacterium is fortified with a dense waxy peptidoglycolipid coating that is impermeable to several antimicrobial compounds [7].

The present study investigated *Mycobacterium tuberculosis* strain common in four areas in CRS using Polymerase chain reaction (PCR) technique.

1. Materials and Methods

A. Study Location

This study was carried out in 4 (Yakurr, Ugep, Calabar South and Calabar Municipal) areas in CRS. Inclusive criteria were GeneXpert positive Male and female from 1-60 years. Sputum samples were cultured in the Tuberculosis Reference laboratory Ibadan. DNA isolation, gel electrophoresis, sequencing, blasting and Polymerase Chain Reaction (PCR) were performed at Molecular Virology Laboratory, University College Hospital (UCH) Ibadan, Oyo State.

B. Sample Collection and Preparation

Sputum samples were collected at the spot from 5 MTB-PH GeneXpert subjects from each area mentioned earlier into 60 mL universal container. These were packed in an ice-cool container and transported immediately to Tuberculosis Reference laboratory Ibadan Oyo State.

Samples were decontaminated by adding an equal volume of 4% NaOH into each container within a falcon tube and incubated for 1-7 hours and vortex for 15 seconds. It was allowed to settle for 15 minutes with the addition of 2 mL of phosphate buffer (6.7 mH, pH 7). Contents were properly mixed and centrifuge at 3,400 g for 15 minutes. The supernatant was discarded and sediment re-suspended with 1 mL sterile phosphate buffer (Key Scientific, Indiana Pathology) to a final volume of 2 mL. The preparation was made for BACTEC MGIT 960 broth culture (BACTEC™ MGIT™).

BACTEC MGIT 960 is a culture tube for broth cultivation of MTB bacilli. The broth preparation usually contains a 7 mL Middle brook 7H9 broth base. The addition of BBL MGIT OADC was added as an enrichment supplement that contain oleic acid, albumin, dextrose, and catalase. Contamination was further reduced by addition of 0.8 mL of reconstituted BBL MGIT PANTA into each culture tube. BBL MGIT PANTA contains an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin. 0.5 ml of processed sputum specimen was inoculated into the culture tube and incubated at 37 °C in a BD BACTEC™ MGIT™ automated mycobacterial detection system. BACTEC- vials without PANTA served as controls. The vials were monitored hourly for one week and daily for 42 days for increased fluorescence. Increased fluorescence with a flashy blue light was an indication of positive MTB in the cultured samples.

C. An Isolation From MGIT Positive Culture Using Conventional Phenol-Chloroform Extraction Technique

About 200 µL aliquots of MGIT positive vials were incubated at 80°C for 1 h to heat kill the mycobacterial cells. Thereafter 150 µL aliquots of MGIT broth were centrifuged at 2000 rpm for 7 minutes. The resulting pellets were re-suspended by the addition of 520 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Further addition of 15 µL of 20% sodium dodecyl sulfate (SDS) and 3 µL of Proteinase K (20 mg/ml) was made and the content was incubated for 1 hour at 37 °C.

After the said period of incubation, 5 M NaCl (100 µl) and 80 µL of a 10% Cetyltrimethyl ammonium bromide (CTAB) in 0.7 M NaCl were added and mixed. The suspension was incubated at 65 °C for 10 minutes and kept on ice for15minutes.An equal volume of phenol chloroform: isoamyl alcohol (24:1) was added to the preparation followed by incubation on ice for 5 minutes and centrifugation at 7200 x g for 20 minute. After centrifugation, the aqueous phase was transferred to a new tube with the addition of cold isopropanol (1:0.6) to maximize

DNA precipitation at –20 ^{0}C for 16 hours or overnight. DNA was collected by centrifugation at 7200 x g for 10 minutes, washed with 500 µL of 70% ethanol, air-dry at room temperature for approximately three hours, resuspend the pellets in 50 µL of TE buffer and kept at 4°C for PCR amplification.

Table 1: Primers used in PCR analysis

	Primers	Oligonucleotide sequence (5' →3')
I.	16s_ f	5'-AGAGTTTGATCMTGGCTCAG-3'
II.	16s_ r	5'-GGTTACCTTGTTACGACTT-3'
III.	RpoB_f	5'TACGGTCGGCGAGCTGATCC-3'
IV.	RpoB_r	5' TACGGCGTTTCGATGAACC-3'

D. PCR Amplification of 16s rDNA *Mycobacterium tuberculosis*

The master mix for 12.5 µL reaction was made comprisedof 0.25 (1 µM) 10 pM offorward 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and Reverse R (5'-GGTTACCTTGTTACGACTT-3') primers, 6.44 µl of sterile distilled H_2O, 0.06 µl of 5 U /µL*Taq* Polymerase, 0.75 (1.5 mM) of 25 mM $MgCl_2$, 0.25 (250 µM) of 10 mM dNTPs, 2.5 µL of green buffer and 2µL of template genomic DNA. The reaction was cycled 35 times as followed: 95 ^{O}C for 5 minutes, (Hot start), 95 ^{O}C for 30 seconds (denaturing); 52 ^{O}C for 45 seconds (annealing) followed by 72 ^{O}C for 30 seconds and final extension of 72 ^{O}C for 5 minutes. The assay was run using a thermocycler and product stored at 4 ^{O}C for further analysis.

E. PCR Amplification of rpoB *Mycobacterium tuberculosis*

Five hundred 500 bp rpoB region that encodes residues 507 to 533 was also amplified using PCR protocol of Lorenz, 2012 [8]. Again, the forward F-(TACGGTCGGCGAGCTGATCC) and reversed R-(TACGGCGTTTCGATGAACC) primers were used for amplification of the rpoB gene. The final expected amplification product of 500 bp fragment was prepared using a master mix of final volume of 12.5 µL. Each PCR mixture contained 2 µl of genomic DNA, 6.44 µl of Sterile distilled H_2O, 2.5 µl of Green buffer, 0.06 µL of 5 U /µL*Taq* Polymerase 0.75 µl of 25 mM $MgCl_2$, 0.25 (250 µM) of 10 mM dNTPs, 0.25 (1 µM) 10 pM forward primer and 0.25 (1 µM) 10 pM reverse primer. Again, the reaction was cycled 35 times with 94 ^{O}C for 60 second (Hot start): 94 s^{O}C for 5 minutes for denaturing, the template was annealed at 57 ^{O}C for 60 seconds and 72 ^{O}C for 60 seconds with final extension of 72 ^{O}C for 10 minutes. The PCR analysis was again run and product stored as mentioned above.

F. Quality Control

Appropriate negative (no DNA control) and positive (MGIT positive process vial content) control was used in each step to identify positive contamination or inhibitor.

G. Electrophoresis

Agarose gel was made by dissolving 1.5 g of agarose gel powder in 100 mL of 1 X TAE buffer and heated in a microwave (RCA 1.1, Walmart) for 5 minutes. 5 µL of MTB-PH amplified PCR product and 1 µLX TAE loading buffer were loading into already prepared 1 % agarose gel submerged with 1 X TAE buffer and turn on at constant voltage of 100 V for 1 hour in the presence of GR Green dye. The Product was sized using 1500 bp DNA ladder for 16s rDNA and 500 bp for rpoB respectively. Products were photographed using a UV trans-illuminator (UVP, USA).

H. Sequencing of Genomic 16s and rpoB Mycobacterium *tuberculosis* genes

The sequence of purified PCR products was determined by Molecular Virology Laboratory University College Hospital Ibadan. DNA sequencing analysis was carried out by a basic local alignment search tool (BLAST) algorithm.

I. Statistical Analysis.

Data collected were analyzed using SPSS version 20 for Descriptive statistics, unpaired T-test for

comparison of the mean range of male and female GeneXpert positive for TB. The level of significance was considered at 90% confidence intervals with a P-value of < 0.05 or α- 0.05.

2. Results and Discussion

The global burden of *M. tuberculosis* infection is overwhelming and continues to remain a significant health problem worldwide [9]. The possibility of achieving the vision of the World Health Organization of ending TB in the nearest future is small. This is because identifying the infecting strains in different areas is neglected. This would have served as a better and easy way of combating the pathogen. The present study report for the first time that the *Mycobacterium strains* infecting people in Yakurr, Ugep, Calabar South and Calabar Municipal are those with 16S rDNA and rpoB gene respectively (**Figure 1 and 2**). Results were compared to types found in other parts of the world. Blasting and alignment of the sequence nucleotides and formation of a phylogenetic tree revealed that the strains were closely related with about 90-100% similarity to strains currently infecting people in the United Kingdom and FARGO while rpoB MTB gene type was also identified to be such common in Belarus, Minsk, and FARGO (**Figure 3 and 4**). The trend of spread of these common MTB strains across the globe may be attributed to free and unchecked human movement from one environment to another.

3. Conclusion:

This study report that MTB strains common in the study areas are type with 16s and rpoB gene composition. This explains partially the reason for high prevalence of MTB rifampicin resistance in our health care facilities. Meanwhile, this study covered just 4 areas in CRS. This calls for more research in other LGAs in and outside CRS for documentation of more scientific data that will be useful for epidemiological survey, non-governmental organizations (NGOs) as well as pharmaceutical companies in developing new lines of effective and more potent drugs.

4. Declaration:

A. Ethical Approval and Consent to Participate

Approval was sought and obtained from Cross River State Ministry of Health, Calabar (Health Research Ethics Committee) before sample collection.

Participants were given brief health education after which consenting individuals were enrolled in the study.

B. Consent for Publication

No details, images or videos relating to any individual were used in this research. Therefore, there is no need for "consent for publication".

C. Availability of Data and Materials

All relevant raw data and information given in this manuscript should be freely available to any scientist wishing to use them for non-commercial purposes. It does not breach the participant confidentiality whatsoever.

CCompeting Interests

The authors declares that there are no competing interests.

E. Funding

This research work was fully funded by the sole author.

Authors' Contributions

The authors have made substantial contributions to the conception, design, analysis, field work, drafting and editing of the work. She has also agreed to be personally accountable for the work.

Acknowledgment

Sincere gratitude goes to the staffs of Tuberculosis Reference, Molecular and Virology Laboratory University College Hospital (UCH) Ibadan Oyo State for their team effort and scientific experience that was of great help in culturing MTB samples, designing Primers, DNA isolation and all other PCR protocols.

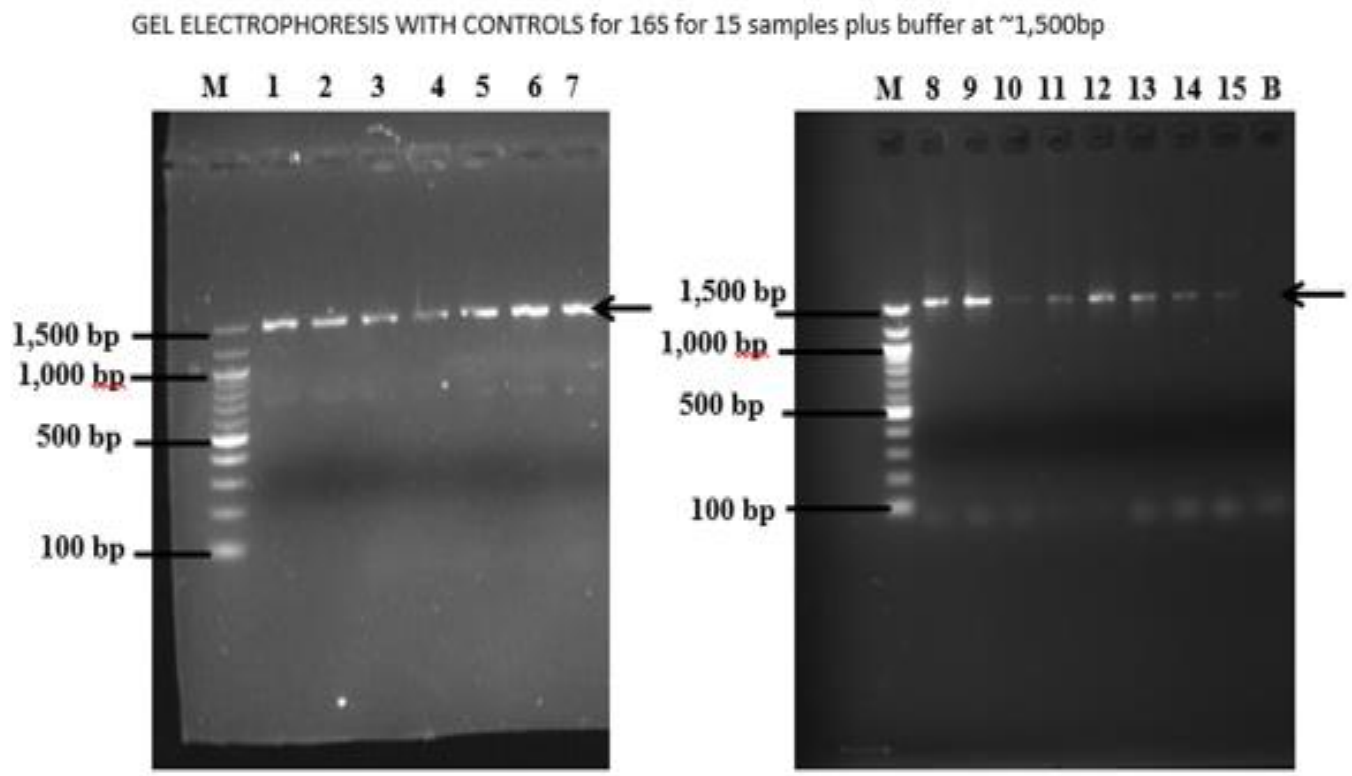

Figure1: Agarosegel electrophoresis 16s ribosomal RNA gene amplified from 15 MTB infected sputum samples from TB patients in CRS. Expected amplicons of about 1,500 bp were produced across all samples (in black arrows). M=100bp molecular ladder. 1 to 15 = samples from patients, B = buffer control.

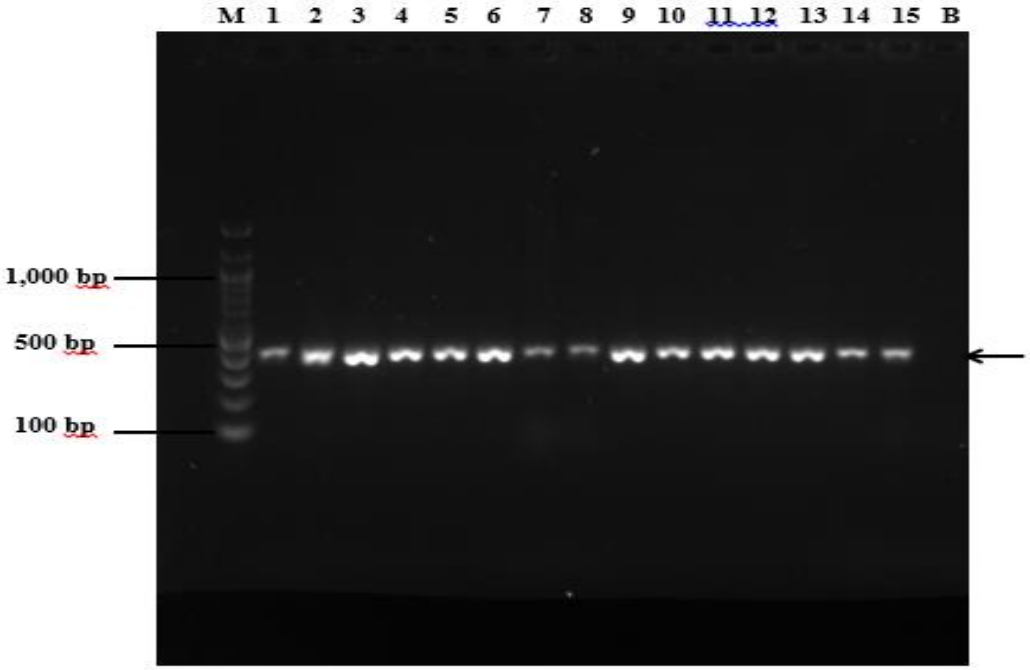

Figure 2: Agarosegel electrophoresis of rpoB gene amplified from 15 MTB infected sputum samples from TB patients in CRS. Expected amplicons of about 400bp were produced across all samples (in black arrows). Lane M=100bp molecular ladder. 1 to 15 = samples from patients, B = buffer control.

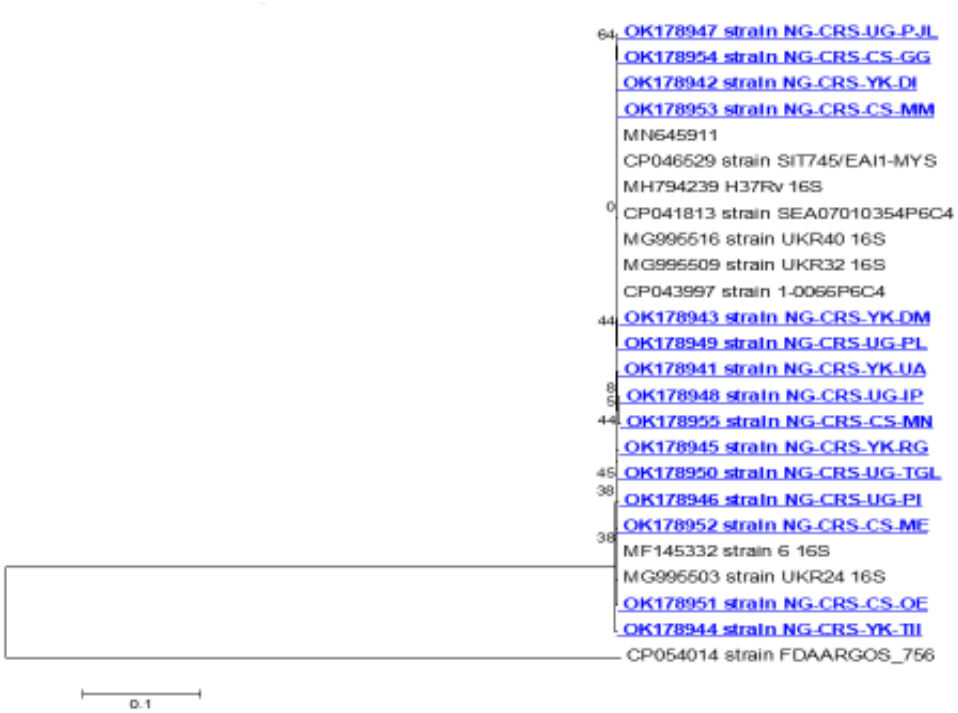

Figure 3: Phylogenetic Tree Showing 16s rDNA in CRS (blue) and other part of the World (black)

Figure 4: Phylogenetic Tree Showing rpoB in CRS (red ink) and other part of the World (black ink)

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APPENDICES

1. SEQUENCEANALYSES

Characteristics and properties of 16S rRNA sequences from fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria using n BLAST on GenBank

Sequence isolate	Origin	GenBank Accession number	Number of nucleotides	Highest nBLAST 0identity (%)	Evalue	Alignment score	Highest query Coverage (%)
NG-CRS-YK-UA	Yakurr	OK178941	1468	99.93	0.0	≥200	100
NG-CRS-YK-DI	Yakurr	OK178942	1468	100.0	0.0	≥200	100
NG-CRS-YK-DM	Yakurr	OK178943	1468	100.0	0.0	≥200	100
NG-CRS-YK-TII	Yakurr	OK178944	1468	99.86	0.0	≥200	100
NG-CRS-YK-RG	Yakurr	OK178945	1468	99.86	0.0	≥200	100
NG-CRS-UG-PI	Ugep	OK178946	1468	99.93	0.0	≥200	100
NG-CRS-UG-PJL	Ugep	OK178947	1468	99.93	0.0	≥200	100
NG-CRS-UG-IP	Ugep	OK178948	1468	99.80	0.0	≥200	100
NG-CRS-UG-PL	Ugep	OK178949	1468	99.93	0.0	≥200	100
NG-CRS-UG-TGK	Ugep	OK178950	1468	99.93	0.0	≥200	100
NG-CRS-CS-OE	Calabar South	OK178951	1468	99.86	0.0	≥200	100
NG-CRS-CS-ME	Calabar South	OK178952	1468	100.0	0.0	≥200	100
NG-CRS-CS-MM	Calabar South	OK178953	1468	99.93	0.0	≥200	100
NG-CRS-CS-GG	Calabar South	OK178954	1468	99.93	0.0	≥200	100
NG-CRS-CS-MN	Calabar South	OK178955	1468	99.80	0.0	≥200	100

Key: NG=Nigeria, MN=Municipal, CS=Calabar South, Yk=Yakurr, UG=Ugep.

2: Percentage pair wise sequence identities of 16Sr RNA sequences from fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria

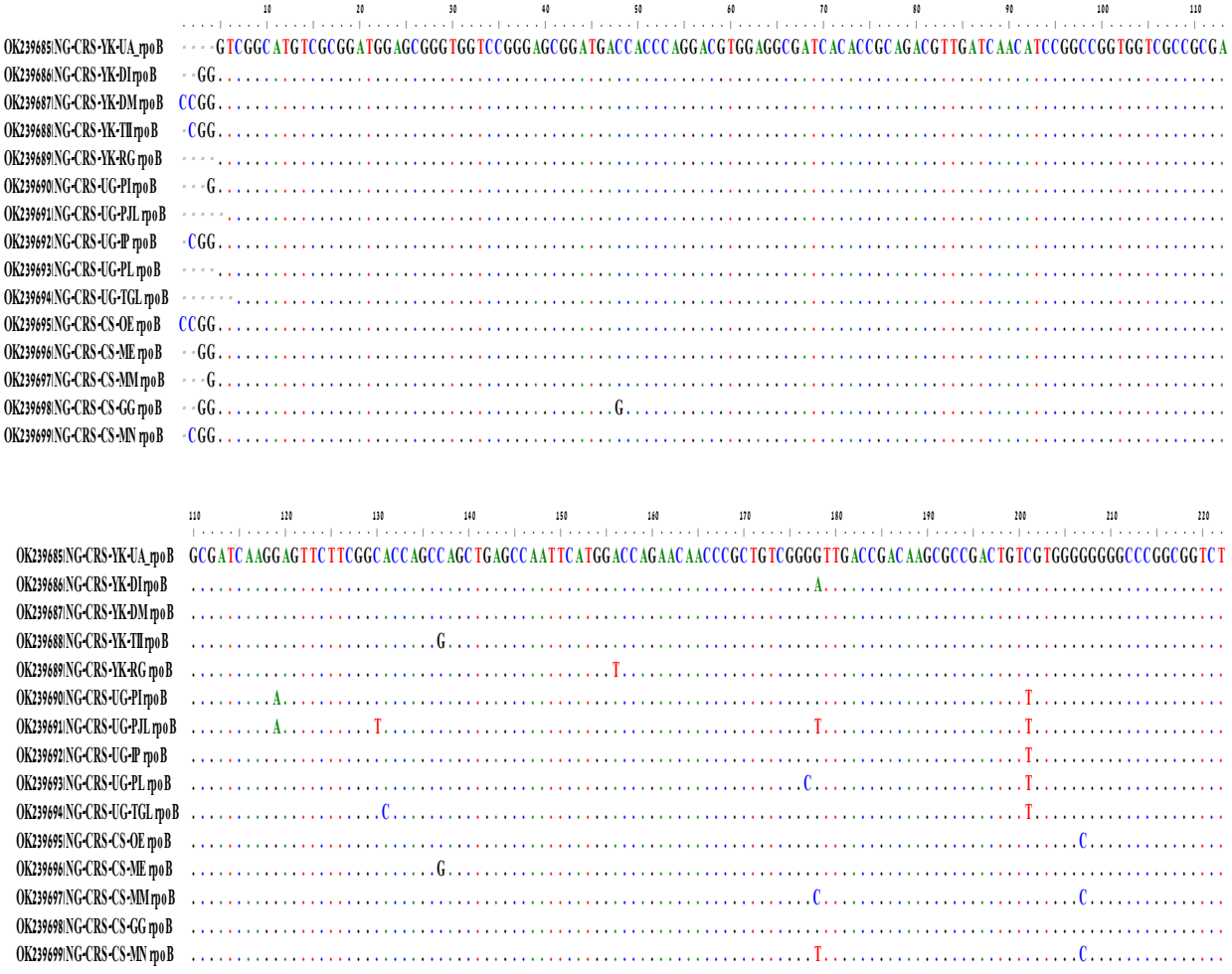
Accession numbers	OK178941	OK1789 42	OK1789 43	OK1789 44	OK1789 45	OK1789 46	OK1789 47	OK1789 48	OK17 8949
OK178941	-								
OK178942	99.93	-							
OK178943	99.80	99.86	-						
OK178944	99.80	99.80	99.66	-					
OK178945	99.73	99.80	99.66	99.59	-				
OK178946	99.73	99.80	99.66	99.59	99.73	-			
OK178947	99.86	99.93	99.80	99.73	99.73	99.73	-		
OK178948	99.86	99.80	99.66	99.80	99.59	99.59	99.73	-	
OK178949	99.86	99.93	99.93	99.73	99.73	99.73	99.86	99.73	-
OK178950	99.80	99.86	99.73	99.80	99.80	99.66	99.80	99.66	99.80
OK178951	99.73	99.80	99.80	99.73	99.73	99.73	99.73	99.59	99.73
OK178952	99.86	99.93	99.80	99.73	99.73	99.86	99.86	99.73	99.86
OK178953	99.86	99.93	99.80	99.73	99.73	99.73	99.86	99.73	99.86
OK178954	99.86	99.93	99.80	99.73	99.73	99.73	100.0	99.73	99.86
OK178955	99.66	99.73	99.73	99.66	99.66	99.66	99.66	99.80	99.80

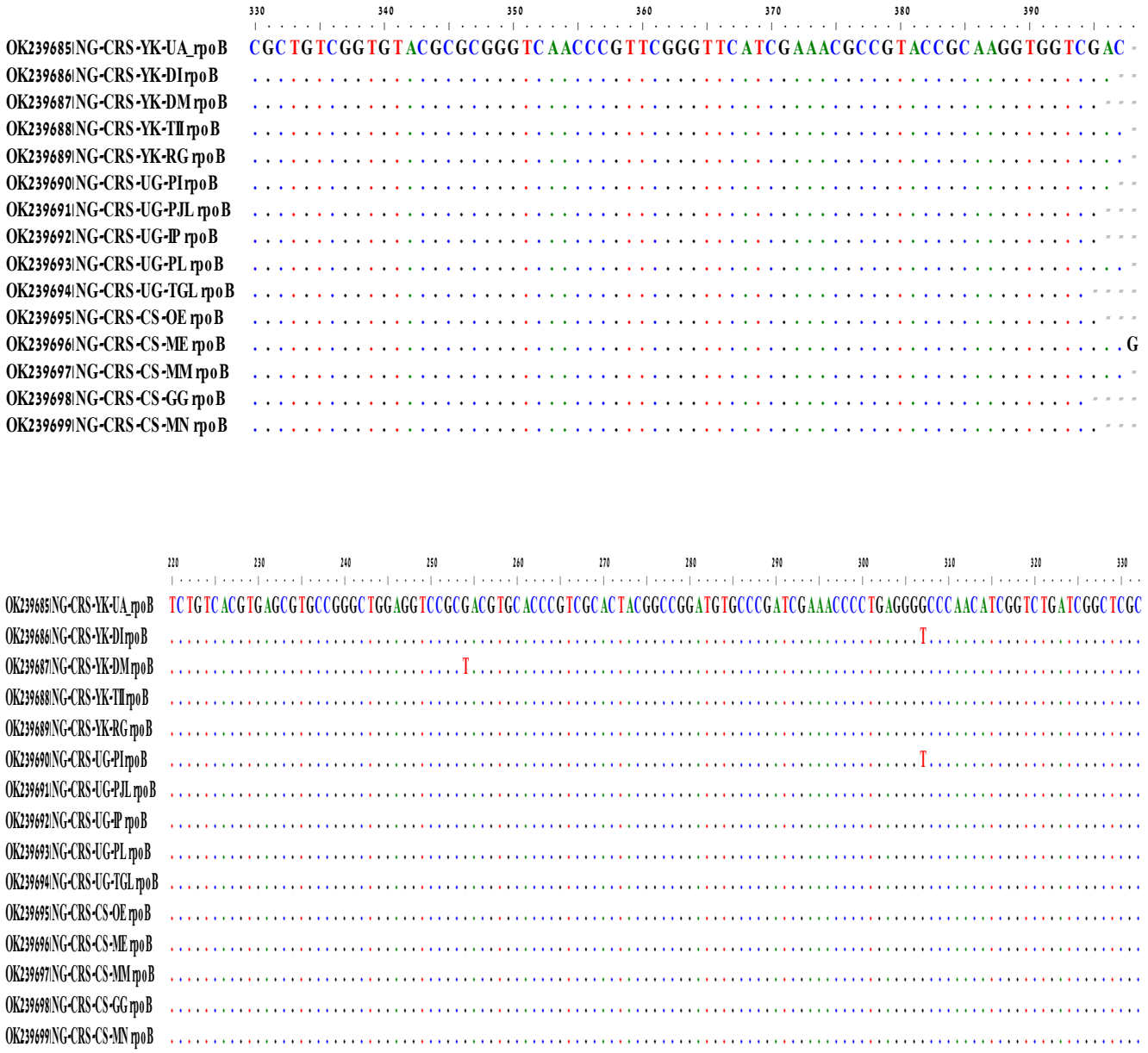
3: Percentage pair wise nucleotide sequence identities of rpoB rRNA sequences from fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria

Accession Numbers	OK178950	OK178951	OK178952	OK178953	OK178954	OK178955
OK178950	-	99.80	99.80	99.80	99.80	99.59
OK178951		-	99.73	99.73	99.73	99.66
OK178952			-	99.86	99.86	99.66
OK178953				-	99.86	99.66
OK178954					-	99.66
OK178955						-

4: Position of Nucleotide Alignment

Nucleotide alignments of partial rpoB gene sequences of fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria.





5: Percentage pair wise identities of nucleotide sequences from rpoB gene amplified from fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria

Accession Numbers	OK239685	OK239686	OK239687	OK239688	OK239689	OK239690	OK239691	OK239692	OK239693	OK239694
OK239685	-									
OK239686	99.49	-								
OK239687	99.74	99.24	-							
OK239688	99.74	99.24	99.49	-						
OK239689	99.74	99.23	99.49	99.49	-					
OK239690	99.23	99.24	98.98	98.98	98.98	-				
OK239691	98.97	98.72	98.72	98.72	98.72	9923	-			
OK239692	99.74	99.24	99.49	99.49	99.49	99.49	99.23	-		

OK239693	99.49	98.98	99.23	99.23	99.23	99.23	98.97	99.74	-	
OK239694	99.48	98.97	99.23	99.23	99.23	99.23	98.97	99.74	99.48	-
OK239695	99.74	99.24	99.49	99.49	99.49	98.98	98.72	99.49	99.23	99.23
OK239696	99.74	99.24	99.49	100.0	99.49	98.98	98.72	99.49	99.24	99.23
OK239697	99.49	99.23	99.23	99.23	99.23	98.73	98.72	99.24	98.98	98.96
OK239698	9974	99.23	99.49	99.49	99.49	98.98	98.71	99.49	99.23	99.23
OK239699	99.49	99.24	99.24	99.24	99.24	98.72	98.97	99.23	98.98	98.96

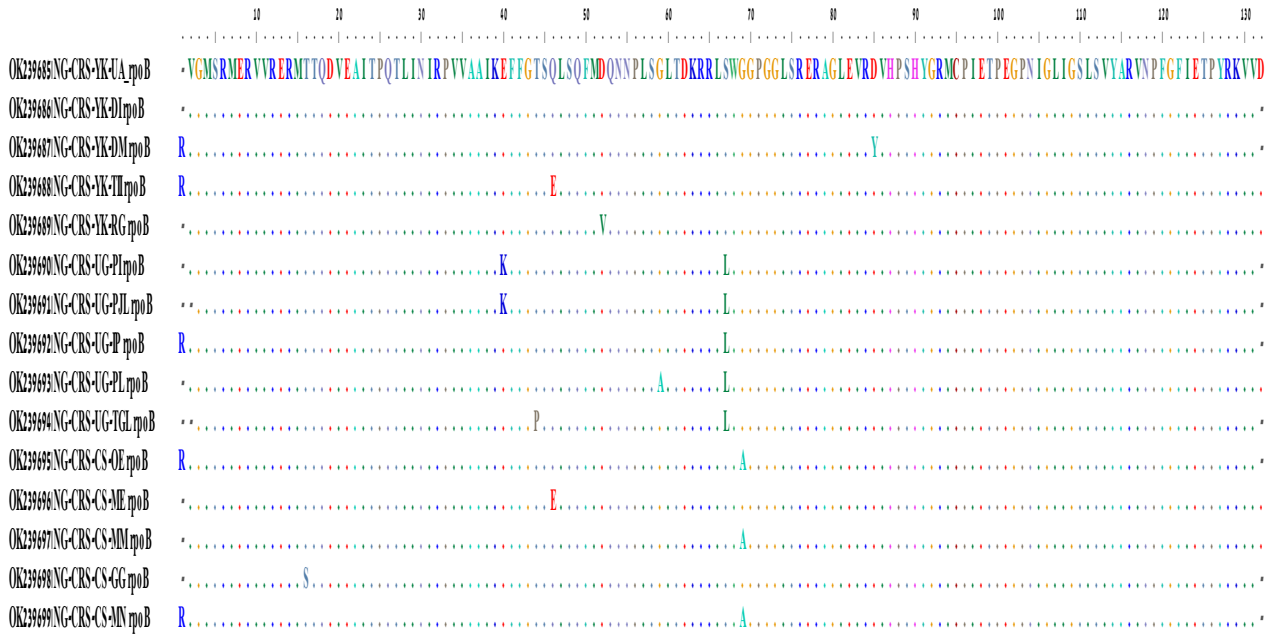
6: Percentage pair wise identities of nucleotide sequences from rpoB gene amplified from fifteen *Mycobacterium tuberculosis* isolates obtained from TB-infected patients in Cross River State, Nigeria.

Accession Numbers	OK239695	OK239696	OK239697	OK239698	OK239699
OK239695	-	99.49	99.74	99.49	99.75
OK239696		-	99.23	99.49	99.24
OK239697			-	99.23	99.74
OK239698				-	99.24
OK239699					-

7: Characteristics and properties of partial rpoB protein obtained from rpoB gene sequences of fifteen *Mycobacterium tuberculosis* isolates obtained from BT-infected patients in Cross River State, Nigeria using nBLAST on GenBank

Sequence Isolate	Region	Number of Amino acids	Highest BLAST p identity (%)	E value	Alignment score	Highest query Coverage (%)
NG-CRS-YK-UA	Yakurr	131	99.21	2e-87	≥200	96
NG-CRS-YK-DI	Yakurr	130	99.21	2e-87	≥200	97
NG-CRS-YK-DM	Yakurr	131	98.43	4e-86	≥200	96
NG-CRS-YK-TII	Yakurr	132	100.0	7e-88	≥200	96
NG-CRS-YK-RG	Yakurr	131	97.71	2e-86	≥200	100
NG-CRS-UG-PI	Ugep	130	97.64	4e-86	≥200	97
NG-CRS-UG-PJL	Ugep	129	97.64	5e-86	≥200	98
NG-CRS-UG-IP	Ugep	131	97.71	9e-87	≥200	100
NG-CRS-UG-PL	Ugep	131	99.21	4e-87	≥200	96
NG-CRS-UG-TGK	Ugep	129	97.64	3e-85	≥200	98
NG-CRS-CS-OE	Calabar South	131	98.47	1e-85	≥200	100
NG-CRS-CS-ME	Calabar South	131	100.0	8e-88	≥200	96
NG-CRS-CS-MM	Calabar South	131	98.47	7e-88	≥200	100
NG-CRS-CS-GG	Calabar South	130	98.43	5e-87	≥200	97
NG-CRS-CS-MN	Calabar South	131	98.47	1e-87	≥200	100

.8: Amino acid alignments of partial rpoB protein obtained from rpoB gene sequences of fifteen *Mycobacterium*



tuberculosis isolates obtained from BT-infected patients in Cross River State, Nigeria

9: Percentage pair wise identities of protein sequences from rpoB gene amplified from fifteen *Mycobacterium tuberculosis* isolates obtained from BT-infected patients in Cross River State, Nigeria

Accession Numbers	OK239685	OK239686	OK239687	OK239688	OK239689	OK239690	OK239691	OK239692	OK239693	OK239694
OK239685	-									
OK239686	100.0	-								
OK239687	99.23	99.23	-							
OK239688	99.24	99.23	98.47	-						
OK239689	99.24	99.23	98.46	98.47	-					
OK239690	98.46	98.46	97.69	97.69	97.69	-				
OK239691	98.45	98.45	97.67	97.67	97.67	100.0	-			
OK239692	99.23	99.23	98.47	98.47	98.46	99.23	99.22	-		
OK239693	98.47	98.46	97.69	97.71	97.71	98.46	98.45	99.23	-	
OK239694	98.45	98.45	97.67	97.67	97.67	98.45	98.45	99.22	98.45	-
OK239695	99.23	99.23	98.47	98.47	98.46	97.69	97.67	98.47	97.69	97.67
OK239696	99.24	99.23	98.46	100.0	98.47	97.69	97.67	98.46	97.71	97.67
OK239697	99.24	99.23	98.46	98.47	98.47	97.69	97.67	98.46	97.71	97.67
OK239698	99.23	99.23	98.46	98.46	98.46	97.69	97.67	98.46	97.69	97.67
OK239699	99.23	99.23	98.47	98.47	98.46	97.69	97.67	98.47	97.69	97.67

10: Percentage pairwise identities of protein sequences from rpoB gene amplified from fifteen *Mycobacterium tuberculosis* isolates obtained from BT-infected patients in Cross River State, Nigeria

Accession Numbers	OK239695	OK239696	OK239697	OK239698	OK239699
OK239695	-	98.46	100.0	98.46	100.0
OK239696		-	98.47	98.46	98.46
OK239697			-	98.46	100.0
OK239698				-	98.46
OK239699					-