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Original Article

# Molecular and Microbiological Assays for Characterization of Multi-Drug Resistant Tuberculosis (MDR-TB) in Clinical Samples- Clinical Relevance for the Disease Diagnosis and Treatment

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ABSTRACT

The ineffectiveness of drugs like isoniazid and rifampicin which are first line of drugs targeted at the treatment of Mycobacterium tuberculosis has increased over the years despite decrease in cases of Mycobacterium tuberculosis either due to the patients not following the right course of treatment or increase in adaptation of the pathogens to the anti-tuberculosis drugs. The aim of this study was the use of Molecular and Microbiological Assays for Characterization of Multi-Drug Resistant Tuberculosis by Multiplex Polymerase Chain Reaction method - Clinical implementation for the Disease Diagnosis and Treatment. 62 clinical samples were collected from symptomatic patients and the processes of decontamination of samples using modified Petroffe method, Ziehl–Neelsen stains (Acid Fast Bacilli Stain, AFB) for initial detection, culturing of clinical samples in Lowenstein-Jensen media, DNA isolation using solid phase (silica column) and magnetic beads methods, Master Mix preparation and PCR set up for multiplex PCR, detection of amplicons using post amplification method and E-gel imager or gel documentation system were followed accordingly. The results of this study showed that out of 62 clinical samples processed, 26(41.93%) stained positive in Acid Fast Bacilli Stain, visible growths were observed on the cultures after 5 weeks and 13(20.97%) came positive in Multiplex Polymerase Chain Reaction (MPCR). Though the initial molecular detection of Mycobacterium tuberculosis is done targeting IS6110 and mpb6 in Nested PCR method, these have never been specific test for detection of drug resistant genes in Mycobacterium tuberculosis Complex (MTC) and therefore ineffective line of drugs may be administered allowing the disease to persist. The use of MPCR is a specific and better way for the diagnosis as well as treatment of Mycobacterium tuberculosis.

**Keywords:** Multi-Drug Resistant tuberculosis, Multiplex PCR, Acid Fast Bacilli Stain, rpoB gene, Mycobacterium tuberculosis.

## 1. INTRODUCTION

Multi-drug-resistant tuberculosis (MDR-TB) is a form of tuberculosis (TB) infection caused by bacteria that are

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resistant to treatment with at least two of the most powerful first-line anti-TB medications (drugs), isoniazid and rifampin. Some forms of TB are also resistant to second-line medications, and are called extensively drug-resistant TB (XDR-TB) [1]. Resistance to anti-TB drugs, a problem recognized in the very early days of the chemotherapeutic era has also emerged as a serious problem [2]. TB drug resistance is characterized by both the types of drugs to which the bacteria lack susceptibility and the manner in which resistance was acquired [3]. Resistance to single agents is the most common type; resistance to multiple agents is less frequent but of greater concern. By convention, "multidrug resistance" is defined as resistance to at least isoniazid and rifampin. [4]

## 2. MATERIALS AND METHODOLOGY

**Sample Collection:** 62 clinical samples from patients who tested positive to *IS6110* gene in Nested PCR in Out-patients and In-patients Departments of Shri Mahant Indiresh Hospital, Patel Nagar, Dehradun, Uttarakhand. **Processing of Samples:** Decontamination of clinical samples for Ziehl–Neelsen stain (Acid Fast Bacilli Stain, AFB) for initial detection and Culturing of clinical samples in Lowenstein-Jensen media, was carried out using (N-acetyl-L-cysteine (NALC), Sodium Hydroxide (NaOH), Tri-sodium citrate, Phosphate Buffer (pH≅ 6.8)) modified Petroffe method. **DNA Isolation:** DNA was isolated using solid phase (silica column) and magnetic beads methods.

Master Mix preparation for 62 reactions, Primer selections and PCR set up for multiplex PCR was done for forward and reverse primers of *rpoB, KatG* and *mabA* genes out as in table 1 and 2

**Table 1: Primer selections for *rpoB, KatG* and *mabA* genes**

| TARGET GENES | PRIMER SET | NUCLEOTIDE SEQUENCE | POSITION | PRODUCT SIZES (bp) |
|---|---|---|---|---|
| ***rpoB*** | PR1(forward)<br>PR2 (reverse) | 5-'CCGCGATCAAGGAGTTCTTC3'<br>5'-CCGCGATCAAGGAGTTCTTC-3' | 1256–1275<br>1570–1551 | 315 |
| ***katG*** | PR3(forward)<br>PR4 (reverse) | 5'GTGCCCGAGCAACACCCACCCATTAC AGAAAC-3'<br>5-TCAGCGCACGTCGAACCTGTCGAG-3' | 1-32 | 2,223 |
| ***mabA promotor*** | PR5(forward)<br>PR6 (reverse) | 5'-ACATACCTGCTGCGCAATTC-3'<br>5'-GCATACGAATACGCCGAGAT-3' | 217 to 198 | 1,362 |

**Table 2: PCR set up for multiplex PCR**

| Segments | Stages | Temperature | Time |
|---|---|---|---|
| | Denaturation | 94ºC | 8minutes |
| S1 | Annealing (35 cycles) | 94 ºC | 30seconds |
| S2 | | 58 ºC | 1:30seconds |
| S3 | | 72 ºC | 1 minutes |
| | EXTENSION | 72 ºC | 7 minutes |
| | FINAL EXTENSION | 4 ºC | 99 minutes |

**Post Amplification:** Post amplification was done using 1.5% Agarose Gel Electrophoresis with 125V of electricity for 12minutes and results were viewed using E-gel imager trans-illuminator and gel documentation system.

## 3. RESULTS

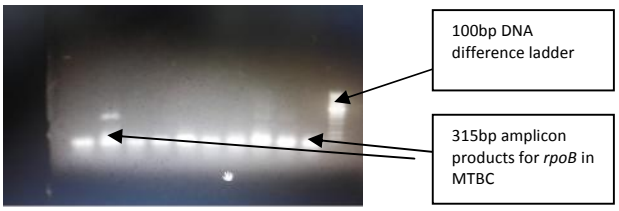


**Fig 1: Post-amplification results for MDRT samples**

For easy and clear presentation of the results, the following abbreviations were used

**AFB** – Acid Fast Bacilli, **MPCR** – Multiplex Polymerase Chain Reaction, **EB-** Endometrial Biopsy, **BAL-** Bronchioaveolar Lavage, **CB-** Cervical Brush, **MB-** Menstrual Blood, **PF-** Pleural Fluid, **BA-** Bronchial Aspirate, **CU**-Caecum Ulcer, **ET-** Endometrial Tissue, **SC** – Subculture, + = Positive, − = Negative

**SAMPLE WISE POSITIVITY AND NEGATIVITY RESULTS**

**Table 3: Sample wise positivity and negativity results**

| S/N | SAMPLE TYPES | CASES | AFB STAINING | | *rpoB* GENE | |
|---|---|---|---|---|---|---|
| | | | POSITIVE | NEGATIVE | POSITIVE | NEGATIVE |
| **1** | PUS | 4(6.45%) | 1(25%) | 3(75%) | 0(0%) | 4(100%) |
| **2** | SUBCULTURE | 1(1.61%) | 0(0%) | 1(100%) | 0(0%) | 1(100%) |
| **3** | TISSUE | 2(6.45%) | 2(100%) | 0(0%) | 0(0%) | 2(100%) |
| **4** | EB | 4(6.45%) | 0(0%) | 4(100%) | 0(100%) | 4(100%) |
| **5** | BAL | 17(27.42%) | 9(52.94%) | 8(47.06%) | 4(23.53%) | 13(76.47%) |
| **6** | CB | 2(3.23%) | 1(50%) | 1(50%) | 1(50%) | 1(50%) |
| **7** | MB | 4(23.53%) | 1(25%) | 3(75%) | 0(0%) | 4(100%) |
| **8** | PF | 1(1.61%) | 1(100%) | 0(0%) | 0(0%) | 1(100%) |
| **9** | BA | 1(1.61%) | 0(0%) | 1(100%) | 0(0%) | 1(100%) |
| **10** | CU | 1(1.61%) | 0(0%) | 1(100%) | 0(0%) | 1(100%) |
| **11** | SPUTUM | 20(32.27%) | 8(40%) | 12(60%) | 5(25%) | 15(75%) |
| **12** | URINE | 4(6.45%) | 2(50%) | 2(50%) | 2(50%) | 2(50%) |
| **13** | SEMEN | 1(1.61%) | 1(100%) | 0(0%) | 1(100%) | 0(0%) |

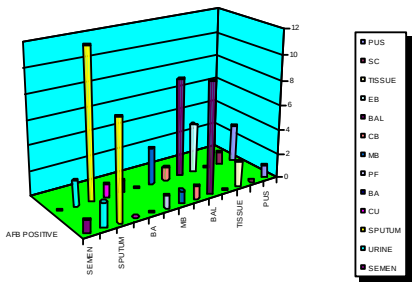


**Chart 1: Positive and Negative cases of samples in Acid Fast Bacilli (AFB) Stain**

As shown in table 6 and chart 1 above, the results from ZN (**Acid Fast Bacilli, AFB)** Staining indicated that of the 62 clinical samples,

**PUS:** Pus was 4(6.45%) of which 1(25%) was positive and 3(75%) stained negatively. **SUBCULTURE (SC):**

Subculture (SC) sample was 1(1.61%) and was 0(0%) positive and 1(100%) negative. **TISSUE:** 2(6.45%) of the samples were tissue derived of which 2(100%) were positive and 0(0%) negative**. ENDOMETRIAL BIOPSY (EB):** 4(6.45%) samples came from endometrial biopsy, 0(0%) coming out positive and 4(100%) negative. **BRONCHIOAVEOLAR LAVAGE (BAL):** BAL was 17(27.42%) sample giving 9(52.94%) positive and 8(47.06%) negative in AFB stain. **CERVICAL BRUSHING (CB):** 2(3.23%) samples came from cervical brushing and 1(50%) was positive while 1(50%) was negative.

**MENSTRUAL BLOOD (MB):** Samples from menstrual blood were 4(6.45%) and 1(25%) stained positive while 3(75%) stained negative. **PLUERAL FLUID (PF):** 1(1.61%) represented PF which was 1(100%) positive and 0(0%) negative: **BRONCHIAL ASPIRATE (BA):** 1(1.61%) was BA, 0(0%) positive and 1(100%) to AFB stain. **CAECUM ULCER (CU):** CU was 1(1.61%) and being 0(0%) positive while 1(100%) negative. **SPUTUM:** Sputum made up the highest cases with 20(32.27) samples; 8(40%) coming positive while 12(60%) came negative. **URINE:** Urine sample were 4(6.45%) giving 2(20%) positive and 2(50%) negative in AFB stain. **SEMEN:** 1(1.61%) semen sample was receive and 1(100%) came positive while 0(0%) negative

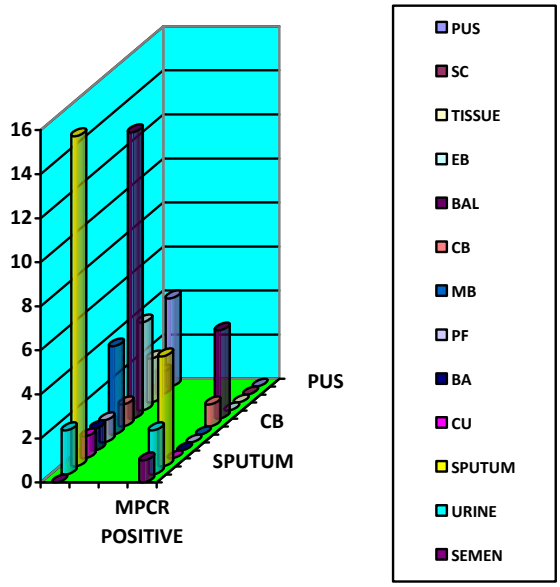


**Chart 2: Positive and negative cases in Multiplex Polymerase Chain Reaction (MPCR)**

On the basis of Multiplex Polymerase Chain Reaction (MPCR), the results obtained from resistant of rpoB gene were:

**Pus** was 4(6.45%) of which 0(0%) was positive and 4(100%) in MPCR. **SUBCULTURE(SC)**: Subculture (SC) sample was 1(1.61%) and was 0(0%) positive and 1(100%) negative. **TISSUE**: 2(6.45%) of the samples were tissue derived of which 0(0%) were positive and 2(100%) negative. **ENDOMETRIAL BIOPSY (EB)**: 4(6.45%) samples came from endometrial biopsy, 0(0%) coming out positive and 4(100%) negative. **BRONCHIOAVEOLAR LAVAGE (BAL)**: BALs were 17(27.42%) sample giving 4(23.53%) positive and 13(76.47%) negative in MPCR assay. **CERVICAL BRUSHING (CB)**: 2(3.23%) samples came from cervical brushing and 1(50%) was positive while 1(50%) was negative. **MENSTRUAL BLOOD (MB)**: Samples from menstrual blood were 4(6.45%) and 0(0%) positive while 4(100%) were negative. **PLUERAL FLUID (PF)**: 1(1.61%) represented PF which was 0(0%) positive and 1(100%) negative. **BRONCHIAL ASPIRATE (BA)**: 1(1.61%) of the clinical samples was BA, 0(0%) positive and 1(100%) to MPCR. **CAECUM ULCER (CU)**: CU was 1(1.61%) and was 0(0%) positive while 1(100%) negative. **SPUTUM**: Sputum made up the highest number of clinical sample cases with 20(32.27%) samples; 5(25%) coming positive while 15(75%) came negative. **URINE**: Urine samples were 4(6.45%) giving 2(20%) positive and 2(50%) negative in MPCR. **SEMEN**: 1(1.61%) semen sample was receive and 1(100%) came positive while 0(0%) negative in MPCR.

## GENDER WISE POSITIVE AND NEGATIVE CASES

**Table 4: Gender wise positive and negative cases**

| GENDER | CASES | AFB STAINING | | *rpoB* | |
|---|---|---|---|---|---|
| | | POSITIVE | NEGATIVE | POSITIVE | NEGATIVE |
| **FEMALE** | 34(54.84%) | 12(35.29%) | 22(64.71%) | 3(8.82%) | 31(91.18%) |
| **MALE** | 28(45.16%) | 14(50%) | 14(50%) | 9(32.14%) | 19(67.86%) |

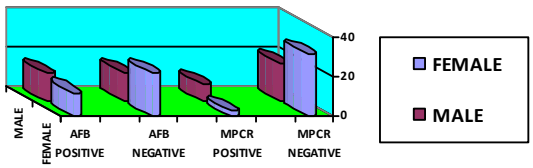


**Chart 3: AFB and MPCR results of male and female samples**

As indicated in table 7 and chart 3, 34(54.84%) of the clinical specimens were from females giving 12(35.29%) positive and 22(64.71%) negative AFB staining while 3(8.82%) were positive to MPCR and 31(91.18%) were negative. 28(45.16%) of the clinical specimens were from males giving 14(50%) positive and 14(50%) negative AFB staining while 9(32.14%) were positive to MPCR and 19(67.86%) were negative.

As indicated in table 7 and chart 3, 34(54.84%) of the clinical specimens were from females giving 12(35.29%) positive and 22(64.71%) negative AFB staining while 3(8.82%) were positive to MPCR and 31(91.18%) were negative. 28(45.16%) of the clinical specimens were from males giving 14(50%) positive and 14(50%) negative AFB staining while 9(32.14%) were positive to MPCR and 19(67.86%) were negative.

### Age Wise Positivity and Negativity Of Samples

**Table 5: Age wise positivity and negativity of samples**

| S/N | AGE RANGE(YEARS) | CASES | AFB STAINING | | *rpoB* | |
|---|---|---|---|---|---|---|
| | | | POSITIVE | NEGATIVE | POSITIVE | NEGATIVE |
| **1** | 00-20 | 8(12.9%) | 2(25%) | 6(75%) | 1(12.5%) | 7(87.5%) |
| **2** | 21-40 | 37(59.68%) | 16(43.24%) | 21(56.76 %) | 10(27.03%) | 27(72.97%) |
| **3** | 41-60 | 15(24.19%) | 7(46.67%) | 8(53.33%) | 2(13.33%) | 13(86.67%) |
| **4** | 61-ABOVE | 23.23%) | 1(50%) | 1(50%) | 0(0%) | 2(100%) |

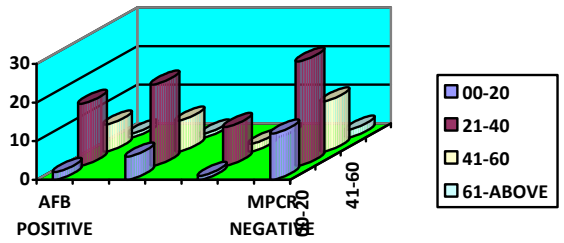


**Chart 4: Age wise positivity and negativity of samples**

Grouping the sample according ages, the range was of 20 years intervals of age as presented below:

**00-20 years** of age were 8(12.9%) of the processed samples, giving 2(25%) positive and 6(75%) negative results in AFB stain while 1(12.5%) was positive and 7(87.5%) were negative in MPCR. Age range between **21-40 years** had the highest number of processed clinical samples making 37(59.68%) of the samples and 16(43.24%) being positive in AFB stain while 21(56.76%) came negative in AFB stain. In MPCR assay, 21(56.76%) were positive while 27(72.97%) came negative. **41-60 years** group were 15(24.19%) of the 62 clinical samples out of which 7(46.67%) were positive and 8(53.33%) were negative in AFB stain with 2(13.33%) positive and 13(86.67%) negative in MPCR. 2(3.23%) samples were receive from the age group **61 and above** with 1(50%) positive while 1(50%) negative in AFB stain whereas 0(0%) positive and 2(100%) negative in MPCR.

### POSITIVITY AND NEGATIVITY BASED ON SITES OF INFECTION

**Table 6: Extrapulomonary Sites**

| CASES 23(37.1%) | POSITIVE | NEGATIVE |
|---|---|---|
| AFB STAINING | 8(34.78%) | 15(65.21%) |
| MPCR | 4(17.39%) | 19(82.61%) |

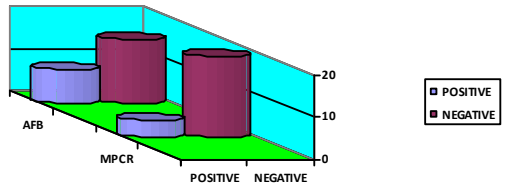


**Chart 5: Results from Extrapulomonary Sites**

Based on site of infection, clinical samples from Extrapulmonary sites were 23(37.1%) out of the 62 clinical samples. 8(34.78%) stained positive in AFB stained while 15(65.21%) were negative in the stain, and on the basis of MPCR, 4(17.39%) were positive while 19(82.61%) gave negative results.

### PULMONARY SITES

**Table 7: Samples Pulmonary Sites**

| CASES 39(62.9%) | POSITIVE | NEGATIVE |
|---|---|---|
| AFB STAININING | 8(20.51%) | 31(79.49%) |
| MPCR | 9(23.08%) | 30(76.92%) |

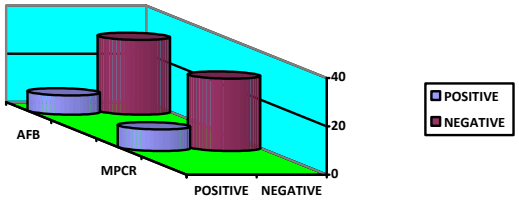


**Chart 6: Results from Pulmonary sites**

Clinical samples from pulmonary sites were 39(62.9%) with 8(20.51%) showing positive results and 31(79.49%) negative in AFB stains. In MPCR, 9(23.08%) were positive while 30(76.92%) came negative to the target genes.

## 4. DISCUSSION

*Mycobacterium tuberculosis* (MTB) as a pathogenic bacteria species in the genus *Mycobacterium* has been causative agent of most cases of tuberculosis, which, in some cases develop drug resistance [5]. The complete or partial resistant to the effects of drugs used for the treatment of tuberculosis by the *Mycobacterium tuberculosis* Complex (MTC) either due to wrong line of drugs prescriptions or the patients not taking proper course of treatment is called multi-drug resistant tuberculosis, MDR-TB. [6]

Amplification technologies offer the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity [5]. Polymerase Chain Reaction has been found to be useful for rapid diagnosis of tuberculosis from variety of clinical specimens. Many laboratories around the globe are using primers designed from *IS6110* sequence of Mycobacterium genome and a more specific and efficient method of Multiplex Polymerase Chain Reaction (MPCR) to ensure accurate line of treatments for tuberculosis patients [6]. *IS6110* is an insertion sequence specific for *Mycobacterium tuberculosis*. The aim of this study was to evaluate the sensitivity and specificity of Polymerase Chain Reaction targeting multiple genes (*rpoB*, *katG* and *mabA*) involved in drug resistance, Microbiological culturing of the clinical samples in Lowenstein-Jensen media and further comparing the results with acid fast bacilli (AFB) stain.

All clinical samples in this study were from patients who were positive to *IS6110 gene* and were undergoing

treatments for which 13(20.97%) had resistant *rpoB gene* in the TB genome thus validating [7] statement that drug-resistant forms of TB can develop if treatment is incorrect or incomplete. This can happen for several reasons, because treatment for TB takes six months and can have difficult side effects; people may be tempted to stop taking their medication before they have completed treatment, particularly if they are starting to feel better. They may be given the incorrect treatment or may fear the stigma of having TB(8). People with infectious drug-resistant TB can then also pass this drug-resistant strain on to others [8]

The findings in this study have corresponded with the reports from World Health Organization (WHO) on the increase in the cases of MDRT since 2013 despite a drop in the number of tuberculosis cases thus creating a global disturbance to the Human race [9]. Multi drug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) are major global health threats. WHO estimates that 480,000 people developed MDR-TB in 2013, leading to 210,000 deaths – but only 45% of cases were identified and treated appropriately. An estimated one in ten cases of MDR-TB has XDR-TB, which had been reported in 100 countries by the end of 2013 [9]. In this study, 13(20.97%) were positive showing an increase in the cases of Multidrug Resistant Tuberculosis when compared to similar study conducted by [6].

*Mycobacterium tuberculosis* affects almost every organ of the body as was observed in this study ranging from **Reproductive System** (Semen, Cervical Brushing, Endometrial Biopsy, Menstrual Blood and Endometrial Tissue), **Circulatory System (**Pus), **Digestive System** (Caecum Ulcer) and **Respiratory System** (Sputum, Bronchioaveolar Lavage(BAL), Pleural Fluid(PF)). Positive cases of drug resistance Mycobacterium tuberculosis using Multiplex PCR were observed in these samples which relates to same study conducted by [1]. Though, the respiratory system is mostly affected because MTB is an aerobic bacterium.

The culturing of Mycobacterium tuberculosis in Lowenstein-Jensen media is one of the ways of detecting the presence of the bacterium in clinical samples between 6-7 weeks of culturing. Though some cultured sample showed significant growth despite showing negative results in MPCR, this was as result of other genes present in the bacterium DNA other than *rpoB*, *mabA* or *katG*. In these cases, genes like *IS6110*, *mpb64* and others may be present.

The prevalence of multi-drug resistance TB is indicated in both males and females (10). Thus, the finding in this study as indicated in table 7 and chart 3, shows that 34(54.84%) of the clinical specimens were from females giving 12(35.29%) positive and 22(64.71%) negative in AFB stain while 3(8.82%) were positive to MPCR and 31(91.18%) were negative. 28(45.16%) of the clinical specimens were from males giving 14(50%) positive and 14(50%) negative AFB staining while 9(32.14%) were positive to MPCR and 19(67.86%) were negative. Thus, despite the female samples being more than the males, the males had more resistant genes than the female due to the fact that the females have a stronger immune system than the males according to [10] who proposed that the biological mechanisms of the X chromosome have a strong impact on an individual's genes, known as genetic imprinting, which gives an immunological advantage to females.

However, according to a study carried out by [5] on Molecular Characterization of *Mycobacterium tuberculosis* using *IS6110* gene with Nested PCR, age group between 21-40 was more affected with TB which was also observed in this study when age range between **21-40 years** had the highest number of processed clinical samples making 37(59.68%) of the samples and 16(43.24%) being positive in AFB stain while 21(56.76%) came negative in AFB stain. In MPCR assay, 10(56.76%) were positive while 27(72.97%) came negative.

Multiplex Polymerase Chain Reaction has shown to be the best method for diagnosis of MDR-TB [11]. In this study, as indicated in the table 9 and chart 5 above, some samples 13(20.97%) came positive in MPCR, 26(41.93%) were positive in AFB stain whereas 23(37.1%) were neither MPCR nor AFB stain positive. Though AFB stain is also one of the ways of detecting MTB, it is disadvantageous because it is a non-specific test and also time consuming which does not show the exact resistant genes present during diagnosis and therefore MPCR is preferable. Despite this, a total of 11(28.21%) out of the 62 samples collected and processed were positive in MPCR as well as AFB stain.

Base on site of infection, clinical samples from Extrapulmonary sites were 23(37.1%) out of the 62 clinical samples. 8(34.78%) stained positive in AFB stained while 15(65.21%) were negative in the stain, and on the basis of MPCR, 4(17.39%) were positive while 19(82.61%) came negative. This, according to similar reports by [12], MTB is not only found in the respiratory system but also found in other parts of the body and have harmful effects too.

Tuberculosis is an aerobic bacterium requiring oxygen for it biochemical and metabolic activities [13]. In this study, it was observed that the pulmonary system is more susceptible to MTB infection because of the aerobic nature of the bacterium as 39(62.9%) of the clinical samples were from the pulmonary system with 8(20.51%) showing positive results and 31(79.49%) negative in AFB stains. In MPCR, 9(23.08%) were positive while 30(76.92%) came negative to the target genes.

## 5. CONCLUSION

Resistance to tuberculosis (TB) drugs is a formidable obstacle to effective TB care and prevention globally. Multidrug-resistant TB (MDR-TB) is multifactorial and fuelled by improper treatment of patients, poor management of supply and quality of drugs, and airborne transmission of bacteria in public places. Case management becomes

difficult and the challenge is compounded by catastrophic economic and social costs that patients incur while seeking help and on treatment.

On this note, the diagnosis of *Mycobacterium tuberculosis* and treatment should be taken seriously in other to reduce the prevalence of Multi-drug Resistant Tuberculosis infection which usually leads to death if not properly handled. Though the initial diagnostic procedure for MTB is usually the *IS6110* gene as target, this routine only gives the positive or negative status of the patients but is not sufficient to detect the presence of resistant gene. The use of Multiplex Polymerase Chain Reaction (MPCR) is the best method for the diagnosis and treatment of *Mycobacterium tuberculosis.*

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