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Are Drug Efflux Genes Present among *Mycobacterium tuberculosis* Isolates from Patients in Lagos, Nigeria?

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Abstract

A major challenge in the treatment of Tuberculosis (TB) is emergence of Multi-Drug Resistant *Mycobacterium tuberculosis* (MDRTB) strains. Efflux genes have been established to be among factors for drug resistance in *Mycobacterium tuberculosis* (*M. tuberculosis*) pulmonary infections by conferring bacterial ability to pump-out drugs from intracellular compartment, making it impossible for drugs to attain intracellular concentration lethal to the organism. There is paucity of data on the role of efflux pump in MDRTB in Nigerian strains of *M. tuberculosis*. Hence, the aim of this study was to detect the carriage, distribution and frequency of efflux pump genes among MDRTB and non-MDRTB isolates from participants with pulmonary *tuberculosis* in Lagos, Nigeria. This study was carried out on *M. tuberculosis* isolated from 1020 participants suspected of pulmonary tuberculosis in Lagos State, Nigeria. A total of 78 *M. tuberculosis* isolates were obtained from the participants suspected of pulmonary tuberculosis. Forty Eight isolates were confirmed as MDRTB and 30 non-MDRTB. Efflux pump genes were investigated in the isolates using the conventional polymerase chain reaction. Statistical analysis was carried out using the Statistical Package for Social Science (SPSS version 20) to compare the efflux pump gene results between MDRTB and non-MDRTB isolates. Different efflux genes types and frequency were detected in MDRTB and non-MDRTB isolates. Carriage of 2 or more alleles of efflux gene types Rv2486c (efpA), Rv2459c (jefA), Rv1877, Rv1002c, Rv0342, Rv2686c and drrC associated with MDR were detected. Additionally, the frequency of efflux genes alleles in MDRTB was significantly different from those in non-MDRTB isolates.

Keywords

Efflux Genes, Alleles, Mutations, MDR, MDRTB, Non-MDRTB, DOTs, Lagos State, Nigeria

1. Introduction

Emergence of drug resistant *M. tuberculosis* strains is a major challenge in the treatment of tuberculosis (TB). Multi resistant mycobacterial strains (MDRTB) and extensive drug resistance (XDR) have been found to be on the increase and that efflux pump mechanism is an important factor responsible for drug resistance [1]. Efflux pumps are transmembrane proteins which actively take part in transporting wide range of substrates including anti-TB drugs from cytoplasm to exterior of cell, thereby nullifying the inhibitory or lethal activity of the drugs (drug resistance). Efflux pump genes have been reported to have different families and classes and impart resistance to broad range of antibiotics like fluoroquinolone, tetracycline, ofloxacin, and isoniazid [2]. Drug efflux pumps prove to be a major challenge for the treatment of MDRTB; they require special attention to understand their functioning to combat the emerging crisis of MDR and finding a better solution for anti-TB therapy.

The relevance of the efflux mechanism to the resistance of clinical strains of *M. tuberculosis* is becoming clearer due to reports of experimental introduction of hypothetical efflux genes into *M. smegmatis* and demonstration of the overexpression of the genes and increases in drug resistance [1] [2]. Other studies [3] showed that the transcription of Rv1258c efflux pump gene in clinical isolates of *M. tuberculosis* increases upon induction by Rifampicin (RIF) and ofloxacin in a clinical multidrug-resistant *M. tuberculosis* isolate. This suggests that efflux pumps are involved in MDR in *M. tuberculosis*. Several mycobacterial efflux pump genes have been characterized using specific markers in laboratory practice [1] [2]. Early studies [4] of drug accumulation in intact cells suggested that drug-resistant mycobacteria accumulated fewer drugs within their cells than the susceptible strains and these results were ascribed to the lower cell envelope permeability in resistant mycobacterial cells. However, higher occurrence of drug efflux genes in drug-resistant strains of mycobacterial cell envelope have been reported [1].

Efflux genes have been detected in *M. tuberculosis* and have also been reported to be responsible for resistance to ant-TB treatment [5]. Bacterial drug efflux pumps have also been classified into five families and these are ATP-binding cassette (ABC) superfamily, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) family, and the multidrug and toxic compounds extrusion (MATE) family [6]. The proteins of the MFS, SMR, RND and MATE families are secondary transporters in which drug efflux is coupled with proton (H1) influx [5] [6]. These pumps are

often referred to as H1-drug antiporters. In contrast, members of the ABC family of multidrug efflux pumps, which are often considered primary transporters, make use of ATP as an energy source [5] [6]. The genome of *M. tuberculosis* contains genes encoding drug efflux transporters from all these families [1]. Active efflux systems, apart from cell wall permeability, provide resistance by extruding the drug molecules that enter the cell [7] [8]. The intracellular concentration of a given drug depends on the balance between its influx and efflux. The processes of drug influx through porins and drug efflux via drug transporters are among distinct and important processes for drug resistance in mycobacteria [8]. The initial role of these pumps is for intracellular survival and high expression is the major contributing factor for developing MDRTB [7] [8]. Several mycobacterial drug efflux pumps have been identified and characterized experimentally [9]. Studies have suggested that efflux systems are often involved with fundamental cellular physiological processes, suggesting that drug extrusion maybe a secondary function [7] [8] [9]. It has been demonstrated by that efflux pump plays a role in three important processes 1) it extrudes and thus provides resistance to several drugs (including rifampicin, one of the most important frontline TB drugs), 2) it is part of the oxidative stress response, and 3) it is needed to maintain normal growth characteristics both on solid medium and in liquid medium [10]. Studies have shown that if Rifampicin resistant strain of *M. tuberculosis* were exposed to Rifampicin, it may activate efflux pump genes thereby compromise the efficacy of second-line drugs containing ofloxacin [11]. It has also been shown that Efflux pump inhibitors (verapamil and reserpine) have the potentials to improve the efficacy of anti-tuberculosis drug treatment [11].

Molecular line probe assay (LPA) technology for rapid detection of multi-drug resistant *Tuberculosis* (MDR-TB) was endorsed by the World Health Organization (WHO) in 2008 due to its advantages [12]. Studies reporting the use of Line Probe Assay (LPA) to improve MDR-TB diagnostic turnaround time were reported [13]. The LPA detects MDR-TB based on the detection of mutations in genes targeted by isoniazid and rifampicin from the DNA sample of sonicated Mycobacterial cells. They include katG, a catalase peroxidase gene involved in the activation of isoniazid. The designated mutant katG in LPA is S315T. The designated mutant inhA in LPA are C15T, A16G, T8C and T8A. For rifampicin, resistance is mediated by mutations in rpoB gene that encoded the beta subunit of *M. tuberculosis* RNA polymerase enzyme. The rpoB mutations included in the LPA DNA strip technology are D516 V, H526Y, H526D and S531L. It has also been reported that though these markers are strongly associated with MDR-TB, M. *tuberculosis* isolates lacking these mutations but eliciting MDR phenotype have been reported by several studies [13] [14]. Studies have also shown that not all rpoB and inhA mutations contribute to multidrug resistance [15]. These two instances have provided the basis for other mechanisms for drug resistance in *M. tuberculosis*. Apart from its rigid and almost impenetrable cell wall of *M. tuberculosis*, the efflux gene system of *M. tuberculosis*

has been shown to be an important alternative mechanism of drug resistance [15].

With the knowledge of bioinformatics, it has been predicted that at least 26 complete and 11 incomplete ATP-binding cassette superfamily (ABC) transporters and 16 Major Facilitator Superfamily (MFS) proteins exist in *M. tuberculosis* [5] and that involvement of several of them in transport of aminoglycosides, fluoroquinolones, chloramphenicol, isoniazid, rifampicin, and tetracyclines has been demonstrated [5]. It has also been shown that the ABC transporters actively pump Rifampicin (RIF) out of the cell thereby lowering the intracellular concentration of RIF to below its binding concentration with the rpoB protein leading to RIF resistance [16]. Expression of 10 different efflux genes in Multi-drug resistant *M. tuberculosis* has also been reported [16]. It was also reported that the concurrent high expression of some efflux pump genes was associated with resistance to the combination of isoniazid and ethambutol [16]. These antibiotics (isoniazid and ethambutol) plus Streptomycin were identified to group together where efflux-mediated drug resistance appears in *M. tuberculosis* and that high expression of efflux genes has also been observed with ofloxacin—a second line anti-TB drug [16]. Resazurin Microtitre Assay (REMA) has been described as for testing MIC of *M. tuberculosis* isolates [16] [17].

Inhibition of efflux by the efflux pump inhibitors reserpine and verapamil leads to an accumulation of RIF within the cell and concurrent binding of RIF to rpoB, leading to inhibition of transcription and cell death and that the evolution of RIF resistance is a dynamic process involving a cascade of adaptive events which leads to a bacterial growth state where hydrophobic compounds are actively extruded from the cell [17]. This has important ramifications for the treatment of RIF resistant TB and supports the need for the development of anti-TB drugs that target both efflux and ATP synthesis to improve the treatment outcome of MDR-TB and XDR-TB [18]. Efflux genes such as p27 & or p55 gene (protein Rv0194) responsible for the extrusion of Rifampicin and iniA gene (protein Rv0342) for extrusion of isoniazid (INH) and ethambuthol (ETM) and other toxic materials out of Mycobacterial cells have been described [19] [20].

The whole genome sequence of *M. tuberculosis* H37Rv has also shown an array of efflux genes with functional activity of extruding INH, RIF, ETM and STR (streptomycin) from the cytoplasm of M. *tuberculosis* or phagosome of infected macrophages to the exterior of the cell [21]. Though different studies have shown the distribution and influence of efflux pump genes in MDRTB [2] [5] [22], there is a paucity of data on the carriage and distribution of efflux pump genes in *M. tuberculosis* strain in Nigeria. Hence; the objectives of this study was to detect the carriage of drug efflux genes, assess the distribution and the frequency of efflux genes among MDRTB and non-MDRTB isolates in Lagos, Nigeria.

2. Materials and Methods

This study was carried out on MDRTB and non-MDRTB isolated from 1020

consented participants suspected of pulmonary *tuberculosis* in 6 selected DOT centres in Lagos State, Nigeria from May 2012 and October 2017. Samples were collected from only participants who voluntarily gave informed consent and were able to submit two consecutive sputum samples. Ethical approval for the study was gotten from the Institutional Review Board of the Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria.

Isolation of *Mycobacterium tuberculosis* on solid medium was done as previously described [23] and on liquid medium using the liquid culture procedure manual. Drug susceptibility testing was carried out using the proportion method procedure. The proportion method was performed as previously described [24]. Anti-mycobacterial drugs were adjusted in the Lowenstein Jenson (LJ) medium to final concentrations of 0.2 - 1 μg/ml for isoniazid (INH), 40 μg/ml for rifampicin (RIF), 4 μg/ml for streptomycin (STR), and 2 μg/ml for ethambutol (ETM). One hundred microliter of prepared bacterial inoculum was inoculated on one each of separate LJ medium with and without drug for test and as a control. This was followed by incubation at 37˚C for 21 - 28 days. Resistance was defined as growth on drug containing tubes greater than 1% of the growth of drug free control medium for INH, RIF, ETM, and 10% for STR [24].

Resazurin Microtitre Assay (REMA) technique was also performed using Middlebrook 7H9-liquid medium. Incubation was done for 14 days and after 7 days 30 μl of 0.02 per cent resazurin sodium salt solution was added to each well and again incubated for further 24 h at 37˚C [17]. A change in colour of the resazurin dye from blue to pink was considered as positive growth and MIC was determined as corresponding to the concentration in the last blue colour in a row. All the experiments were performed in duplicates. Isolates with MICs of INH < 0.25 μg/mL and RIF ≤ 1 μg/mL were defined as being sensitive to INH and RIF, respectively [24].

DNA extraction for molecular assays was carried out as follows: One ml of MGIT 960 culture of reference or clinical *M. tuberculosis* isolates was heated with 40 mg Chelex-100 (Sigma-Aldrich, St. Louis, MO, USA) at 95˚C for 20 min and then centrifuged at 12,000 × g for 15 min. For a PCR, 2 μl of supernatant was used as a source of DNA. The forward and reverse primers for each of the targeted efflux genes used in this study were designed using the DNA sequences of associated genes in the genome of H37Rv [25]. The primers were produced as 20 - 26-mer oligonucleotides with an annealing temperature of 60% - 65% and %GC of 55% - 60%.

Efflux gene detection was done by polymerase chain reaction (PCR). The PCR was set up in a 25 μl reaction volume for amplification of whole gene with 1X CERTAMP buffer, 1.5 units of CERTAMP enzyme (BioTools, Spain), 800 μM dNTP mix (Bangalore Genei, India), 3 mM $MgCl_2$ (Bioron GmbH, Germany), 1.5 μM each of forward and reverse primers and 2 μlof DNA (boiled and snap chilled). The cycle parameters of PCR included initial denaturation at 94˚C for 5 min followed by 35 cycles of 94˚C for 1 min, 60˚C - 62˚C for 30 sec and 72˚C for

2 min, followed by a final extension of 5 min at 72˚C. The 60˚C primer annealing temperature was used for the amplification of Rv2459c (jefA), Rv2686c. Rv2486c (efpA), Rv 0342, drrC, Rv1002c and Rv1877 efflux genes. The PCR products obtained after amplification were separated by horizontal agarose gel electrophoresis.

Agarose gel electrophoresis was used for the efflux genes separation and detection after PCR on 2% (w/v) agarose gel pre-stained with ethidium bromide at 0.5 ug/mL. Gels were run in Tris Acetate EDTA (TAE) buffer (40 mMTris, 2 mM sodium acetate 1 mM Na_2 EDTA, pH 8.0) for 1 h. after electrophoresis, separated efflux genes were detected under UV light and photographed using a Thermo Fischer Gel documentation system (Thermo Fisher, USA). The 100 – 500 molecular sizes of the amplicon extracted were based on mobility of control DNA ladder (Invitrogen, Life Technologies Ltd., Paisley, UK).

Data obtained were entered into Microsoft excel 2007 version and Epi Info version 6.1. Statistical Package for Social Science (SPSS version 20) were used to obtain descriptive and inferential statistics from the data and summarized as frequency and percentages (%) as well as mean + standard deviation (SD). Chi (X2) square of Fischer Exact (when frequency (n) < 5) test was used to investigate the occurrence and frequency and significance of the targeted efflux genes in the MDR TB and the non-MDRTB isolates.

3. Results

Efflux pump genes of different families and classes were detected in the isolated MDRTB (n = 48) and non-MDRTB (n = 30) strains from the study participants as shown in **Figures 1-6**.

The detected efflux pump genes in MDRTB genome were Rv2486c (efpA), Rv1877, Rv1002c, Rv2686c, drrC, Rv2459c (jefA for extrusion of isoniazid and ethambutol-INH/ETM), iniA (Rv 0342 for extrusion of INH and ETM). Carriage of 2 or more alleles of efpA efflux gene and Rv1877, Rv 1002c, Rv0342, Rv2686, drrC efflux gene alleles were associated with multidrug resistance to anti-TB drugs. The efpA efflux gene alleles were detected with MDRTB isolates accounting for 79.7% of the alleles while non-MDRTB accounted for 23.3% (p =0.018). This showed a significant difference between MDRTB and non-MDRTB. On the whole, carriage of 2 or more alleles of efpA efflux gene was associated with multidrug resistance to anti-TB drugs (**Table 1**, **Figure 2**).

Rv1877 alleles were detected (**Table 1**, **Figure 1**) with MDR *M. tuberculosis* isolates accounting for 68.8% of the alleles and non-MDRTB accounted for 20.0% (p = 0.013). There was a significant difference in the carriage of Rv1877 efflux gene alleles between MDRTB and non-MDRTB isolates. This therefore, showed that the carriage of the efflux genes is associated with MDRTB. The Rv2459c (jefA) efflux gene alleles were detected in the isolates with MDR *M. tuberculosis* isolates accounting for 68.8% of these alleles and 30.0% for non-MDRTB (p = 0.007). This showed significant difference between MDRTB and non-MDRTB

Table 1. Efflux gene types function, allelic distributions and carriage frequency in MDRTB and non-MDRTB.

Efflux gene types	Class and Expected functions	Number positive in MDRTB N = 48	Number positive in non-MDRTB N = 30	% Carriage in MDRTB	% Carriage in non-MDRTB	p-value
Rv 1877	MFS. For efflux of drugs	33	33	68.8	20.0	0.013
Rv 2486c (efpA)	MFS. For efflux of drugs eg RIF and INH	38	38	79.7	23.3	0.018
Rv 0342(iniA)	Accessory protein. Efflux of INH, ETM and other toxic materials	32	32	66.7	33.3	0.024
Rv 2686c	ABC. Accessory protein for drug efflux	31	31	64.6	36.7	0.025
drrC	ABC. For efflux of drugs eg RIF	27	27	56.3	46.6	0.027
Rv 2459c(jefA)	MFS. For efflux of INH and ETM	33	33	68.8	30.0	0.007
RV 1002c	Accessory protein for efflux of drugs	30	30	62.5	40.0	0.017

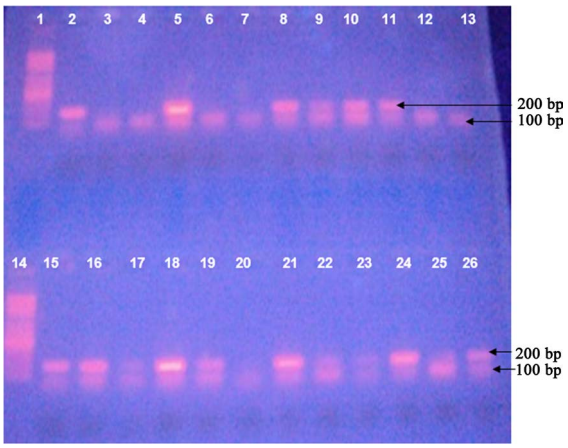

Figure 1. Rv1877 efflux pump allele profile between rifampicin mono resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 2, 5, 8, 10, 15 - 21 (MDR *M. tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162) Lanes 3, 4, 6, 7, 9, 11 - 14, 22 - 26.

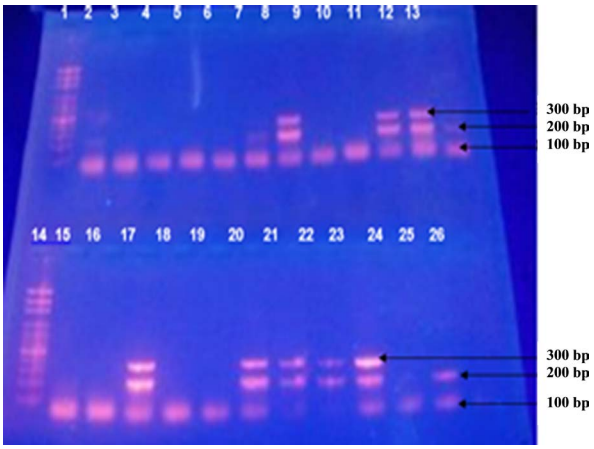

Figure 2. *efpA*(Rv2486c) efflux pump allele profile between rifampicin resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 8, 11, 12, 17, 20 - 23 (MDR M. *tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162, 185) Lanes 2 - 7, 9, 10, 13 - 18, 24 - 25 (Rifampicin resistant isolates: 008, 028, 037, 041, 046, 052, 055, 058, 066, 075, 079, 082, 087, 112, 144, 150).

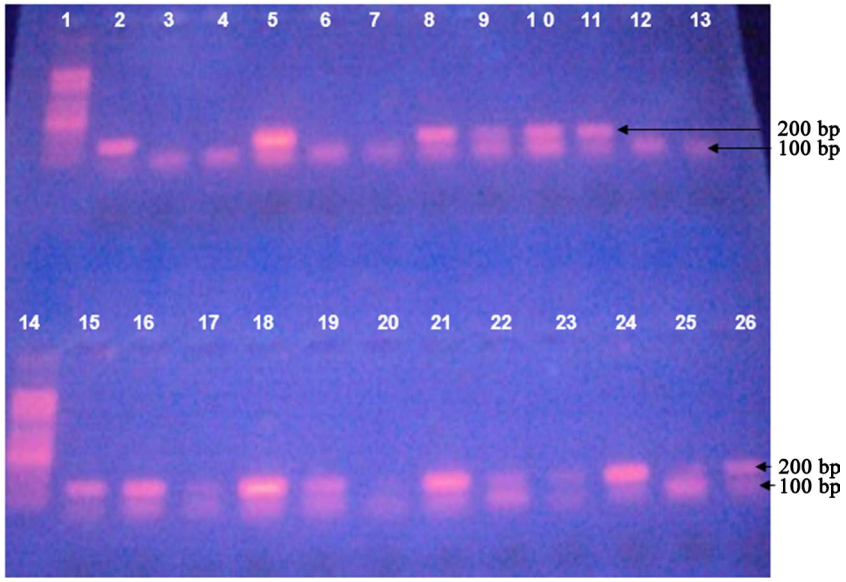

Figure 3. *iniA*(Rv2686c) efflux pump allele profile between rifampicin resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 8, 11, 12, 17, 20 - 23 (MDR *M. tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162, 185) Lanes 2 - 7, 9, 10, 13 - 18, 24 - 25 (Rifampicin resistant isolates: 008, 028, 037, 041, 046, 052, 055, 058, 066, 075, 079, 082, 087, 112, 144, 150).

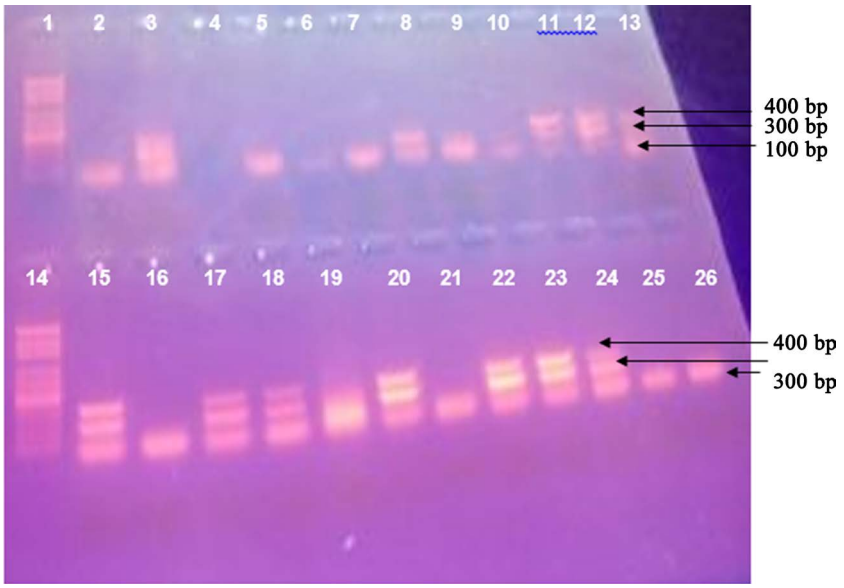

Figure 4. drrC efflux pump allele profile between rifampicin mono resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 8, 11, 12, 17, 20 - 23 (MDR *M. tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162, 185) Lanes 2- 7, 9, 10, 13 - 18, 24 - 25 (Rifampicin resistant isolates: 008, 028, 037, 041, 046, 052, 055, 058, 066, 075, 079, 082, 087, 112, 144, 150).

and that Rv2459c (jefA) efflux gene allelic carriage frequency is associated with MDRTB (Table 1, Figure 5).

Rv1002c efflux gene alleles were detected in the isolates with MDR *M. tuberculosis* isolates accounting for 62.5% of the alleles and 40.0% (p = 0.017).

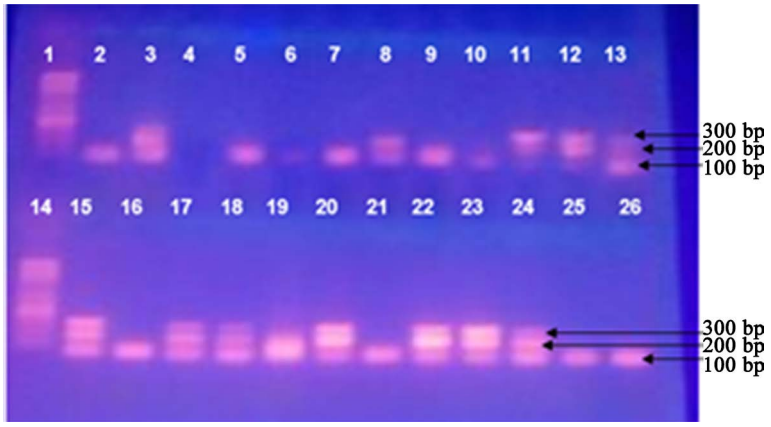

Figure 5. jefA (Rv2459c) efflux pump allele profile between rifampicin resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 2, 5, 8, 10, 15 - 21 (MDR *M. tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162) Lanes 3, 4, 6, 7, 9, 11 - 14, 22 - 26.

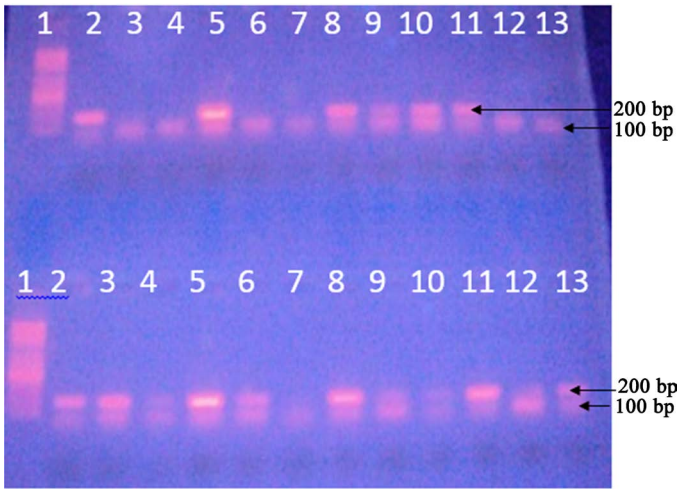

Figure 6. Rv1002c efflux pump allele profile between rifampicin resistant and multidrug resistant *M. tuberculosis* isolates. Lanes 1 & 14 = 100 bp DNA ladder marker; Lanes 2, 5, 8, 10, 15 - 21 (MDR *M. tuberculosis* isolates: 015, 038, 072, 105, 133, 147, 162) Lanes 3, 4, 6, 7, 9, 11- 14, 22 - 26.

This showed significant difference in the MDRTB and non-MDRTB isolates and that carriage of Rv1002c efflux gene alleles was associated with MDR TB (Table 1, Figure 6). Rv0342 efflux gene alleles were detected in the isolates with MDR *M. tuberculosis* isolates accounting for 66.7% of the alleles and non-MDRTB accounting for 33.3% (p = 0.024). This showed that carriage of Rv0342 was associated with multidrug resistance (Table 1). The Rv2686c efflux gene alleles were detected in MDR *M. tuberculosis* isolates, accounting for 64.6% of the alleles and 36.7% for non-MDRTB isolates (p = 0.025) as shown in Table 1. The significant difference observed in the abundance of this type of efflux genes in MDRTB and non-MDRTB showed that carriage of Rv2686c was associated with multi drug resistance. The drrC efflux gene alleles were detected in MDR *M. tuberculosis* isolates accounting for 56.3% of the alleles and

non-MDRTB was 46.6% (p = 0.027) as shown in **Table 1**, **Figure 4**. It also showed that was significant difference in allelic abundance in the carriage of drrC efflux genes between MDRTB and non-MDRTB isolates and is therefore associated with multidrug resistance.

4. Discussion

Efflux gene types responsible for extrusion of isoniazid (INH), ethambutol (ETM) and Rifampicin (RIF) were detected in the MDRTB and non-MDRTB isolates in this study. This finding is similar to those previously reported in mycobacterial isolates [19] [20] [22]. There was significant difference in allelic abundance of mutation determinants in MDRTB and non-MDRTB (60.6% - 79.7% in MDRTB vs. 20.3% - 39.4% in susceptible *M. tuberculosis*).

MDR-TB rate of 19.4% and 15.8% by proportion method and Genotype MTBDR Plus assay respectively were found in this study, suggesting that the difference may be due to efflux genes as another mechanism of resistance. Other efflux pump genes in MDRTB genome detected were Rv2486c (efpA), Rv1877, Rv1002c, Rv2686, drrC and Rv2459c (jefA). They were found to be significantly associated with multidrug resistance ($P < 0.05$). This finding is similar to the study conducted by [5] and [16] where it was reported that at least 26 complete and 11 incomplete ATP-binding cassette superfamily (ABC) transporters and 16 major facilitator superfamily (MFS) proteins exist in *M. tuberculosis* and reported expression of 10 different efflux genes in *M. tuberculosis* respectively. The results in this studies agreed with those of [19] [20] [22] who reported that efflux gene p27 & or p55 gene (protein Rv 0194) is used for pumping-out (extrusion) of Rifampicin and (b) iniA gene (protein Rv0342) for extrusion of INH and ethambuthol. This findings also agreed with the studies by [11] which showed that efflux genes such as Rv 2994, Rv 1877, Rv 1258, Rv 2333c and many others were expressed in the genome of some strains of *M. tuberculosis* and that the efflux genes were involved in efflux and transport of anti TB drugs especially rifampicin and Isoniazid (INH). They also reported that the efflux pump genes in *M. tuberculosis* vary with strains depending on the genetic backgrounds of the strains isolated.

The danger in exposing rifampicin-resistant strains of *M. tuberculosis* to rifampicin therapy had been reported [11]. Rifampicin-resistant strains of *M. tuberculosis* exposed to Rifampicin, caused a reduction in susceptibility to ofloxacin (a second-line anti TB drug) to above the critical concentration of 2μg/ml which is used to define resistance to the drug [11]. The lesson from this is that all rifampicin resistant patients must be screened for the presence of efflux pump genes by molecular methods before exposing the patients to rifampicin therapy to avoid creating resistance to even the second-line anti TB drugs. It is therefore important to determine resistance to rifampicin either by detection of mutation markers of rifampicin resistance or by detection of efflux genes targeted at rifampicin in *M. tuberculosis* causing infection in a particular patient before the

commencement of anti-TB regimens. This is to avert raising strains that will be resistant to second line anti-TB drug such as ofloxacin [16] [17].

More than 8 efflux gene of different families, classes and functions were observed in this study. This finding is similar to the study conducted by [5] and [16] where it was reported that at least 26 complete and 11 incomplete ATP-binding cassette superfamily (ABC) transporters and 16 major facilitator superfamily (MFS) proteins exist in *M. tuberculosis* and reported expression of 10 different efflux genes in *M. tuberculosis* respectively. However, there is need to demonstrate loss of resistance by MDRTB using efflux gene inhibitors reported by [5] and [16] to confirm or refute reports that specific inhibitors of efflux pumps (verapamil and reserpine), inhibit efflux systems in *M. tuberculosis* and produced an increase in susceptibility to antibiotics [20].

Results of this study also showed that the level of streptomycin, isoniazid, rifampicin and ethambutol resistance varies independently of the mutation in the rpo B gene. This also suggested that other biological mechanisms such as efflux pumps were responsible for resistance even where mutation of the rpoB gene did not occur in the *M. tuberculosis*. It has been shown that Efflux pump inhibitors (verapamil and reserpine have the potentials to improve the efficacy of anti-*tuberculosis* drug treatment [11]. It had also earlier been reported that specific inhibitors of efflux pumps, verapamil and reserpine, exposure of bacteria to substances that inhibit efflux systems produces an increase in susceptibility to antibiotics [19]. The findings in this study further confirm what bioinformatics knowledge has shown that there is expression of over 10 different efflux genes in Multi-drug resistant *M. tuberculosis* [16] and at least 26 complete and 11 incomplete ATP-binding cassette superfamily (ABC) transporters and 16 major facilitator superfamily proteins exist in *M. tuberculosis* [16] [17]. It was also reported that the simultaneous over-expression of some efflux pump genes was associated with resistance to the combination of isoniazid and ethambutol [17]. These antibiotics (isoniazid and ethambutol) plus Streptomycin were identified to group together where efflux-mediated drug resistance appears to be important in *M. tuberculosis*. Over expression of efflux genes has also been observed with ofloxacin—a second line anti-TB drug [17].

5. Conclusion

Different efflux gene types and functions were detected in the MDR *Mycobacterium tuberculosis* isolates from this study. The multiplicity of efflux gene alleles was seen more in MDRTB isolates than in non-MDRTB. Further studies to investigate the possible inhibition of the detected efflux genes using beta blockers such as verapamil and reserpine are recommended.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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