Validity of D29 Phage Method in the Detection of Multi Drug Resistant *Mycobacterium tuberculosis* in Sudan

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Abstract: Tuberculosis is still one of the major health problems worldwide, with high mortality and morbidity. While third of the world population is infected with TB bacilli, the situation is getting worse by the rising emergence of multi drug resistant strains of *Mycobacterium tuberculosis* (MDR-TB). Conventional method for drug susceptibility testing requires months before results can be reported. However, rapid methods such as phage assay have been developed and recorded as useful tools for more rapid diagnosis.This study is a descriptive cross-sectional laboratory based study which aimed to evaluate the usefulness of phage assay compared to proportional method and PCR in the diagnosis of MDR TB.Sputum specimens were collected from ninety (n=90) acid fast bacilli consented patients, and were processed for direct D29 and culture. All successful cultured isolates were subjected to biochemical tests for phenotypic characterization and further genotypic confirmation was made by amplification of *IS 6110*. For drug susceptibility testing, proportional method was adopted followed by both indirect D29 and amplification of rpoB gene.The results showed that 21/90 (23.3%) of the specimens were identified as rifampicin resistant by direct D29 method, 75/90 (83.3%) of the specimens showed growth on LJ medium similar to MTB complex colonies while 5/90 (3.3%) were tentatively identified as rapid growers. 60 out of the 75 slow growers (80%) were confirmed as MTB complex members depending on their biochemical and molecular characters (yielding a band of 123 bp of *IS 6110*). DST results for the 60 MTB isolates showed that 31/60 were drug resistant and that isoniazid composed for the highest percentage of resistance (20/31), followed by rifampicin (19/31) while MDR was detected in 18/60 of the isolates. Furthermore 15/60 were confirmed as rpoB positive.The study highlighted the high prevalence of MDR TB in Kassala State. Also the use of D29 phage method in its first trial of application in Sudan revealed high sensitivity and specificity, which when added to its major character of time saving, can represent a reliable method for detection of MDR.
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1. Introduction

Tuberculosis (TB), though curable, still remains a major public health concern worldwide. According to the report of the World Health Organization, there is at least one new infection with TB bacilli every second. Overall, one-third of the world's population is currently infected with the TB bacillus (1.86 billion people). 5-10 % of people who are infected with TB bacilli (but who are not infected with HIV) become sick or infectious at some time during their life. People with HIV and TB co- infection are much more likely to develop TB. Approximately, 8.8 million new cases occur each year, resulting in 2- 3 million deaths around the world [1]. The situation of TB is made even worse by the rising emergence of drug resistant strains of *Mycobacterium tuberculosis* [2]. Multidrug-resistant (MDR) tuberculosis is defined as disease caused by strains of *M. tuberculosis* that are at least resistant to treatment with isoniazid and rifampicin. MDR tuberculosis and XDR tuberculosis are serious threats to the progress that has been made in the control of tuberculosis worldwide over the past decade [3]. An estimated 4.8% of all new and previously treated TB cases diagnosed worldwide in 2006 were MDR TB [4]. Because patients with MDR TB are resistant to treatment with first-line drugs, they could be treated with second-line drugs that are more expensive, have more side effects, often require injection, and involve longer treatment.

Conventional methods for susceptibility testing require several months before results can be reported. However, rapid methods to determine drug susceptibility have been developed recently. Phage assay have been reported as a rapid useful tool for antimicrobial susceptibility testing [5].

2. Materials and Methods

Ethical Clearance

This study was approved by the National Ethics Committee, Ministry of Health, Sudan. Written consent was obtained from every patient before being enrolled in the study.

Study Design and Collection of the Samples

This study is a descriptive cross sectional laboratory based study. It was conducted during the period from August 2009 to January 2012 in different hospitals in Kasala state of Sudan including Kassala Educational Hospital, Khashm Elgerbah Hospital, Wager Hospital, Aroma Hospital, Phatou Primary Health Care Center and West El-Gash Primary Health Care Center. Ninety patients (n= 90) with positive acid fast bacilli (AFB) smears attending the above mentioned were enrolled in this study, after being given their informed consent. Sputum samples were collected in clean, wide mouthed, and leak proof containers according to WHO guidelines [6]. Each patient was clearly instructed on how to produce an adequate sputum specimen, the importance of sputum examination for diagnosis or follow-up of TB, how to open and close the containers and the need for collecting real sputum, not saliva.

Phenotypic Characterization

Ziehl – Neelsen (ZN) staining was done as described by Somoskovi *et al*. (2001) and Van Deun*et al.* (2005) [7,8]. On the other hand, Lowenstein Jensen medium slope was inoculated with 20 µl of the sediment that was obtained from the digestion and decontamination of the sputum sample. Further identification of mycobacteria was done following Kent and Kubica method (1985) [9].

Drug Susceptibility Test (DST)

Antibiotic susceptibility test was done for all isolates, an amount of 0.1ml of 10^{-4} bacillary suspension was inoculated onto two slopes of LJ medium as control number two, concentration of 10^{-2} bacillary suspension was also inoculated onto two slopes of LJ media as control number one, two slopes of LJ containing streptomycin 8.0 µg/ml (SM), two slopes containing rifampcin 40.0 µg/ml (RIF), two slopes containing isoniazid 0.2 µg/ml (INH) and two slopes containing ethambutol 2.0 µg/ml (EMB) were inoculated with a suspension of mycobacteria containing 10^{-2} bacilli. Cultures were examined regularly up to six weeks for the presence or absence of growth.

Genotypic Characterizations

Polymerase chain reaction was used to confirm the results of the conventional methods according to Persing *et al*.,1993 [10].

DNA Extraction

DNA was extracted by boiling method; 3-5 loop full of mycobacterial colonies were harvested in 500 ul double distilled water in sterile eppendrof tube, boiled in water bath at 100° C for 10 minutes, then centrifuged in 12000 rpm for 5 minutes, the supernatant was collected in sterile eppendorf tube and stored in -20°C until used as template for PCR.

Primers of Insertion Sequence *IS6110*

Amplification of insertion sequence *IS6110* (123 bp) (Eisenach *et al.*, 1990) [11]was performed with a set of primers having the following sequence:

Forward: `5 (CCTGCGAGCGTAGGCGTCGG) `3

Reverse: 5` (CTCGTCCAGCGCCGCTTCGG) `3

Primers of *rpoB Gene*

Detection of rifampicin resistant strains was conducted by amplifying rpoB gene to produce amplicon of 193 bp according to Tavakoli *et al*., (2005) [12].

Upstream primer *rpo*105 (5′-CGTGGAGGCGATCACACCGCAGACGT-3′) and Downstream primer *rpo273* (5′-GACCTCCAGCCCGGCACGCTCACG-3′).

Fast Plaque-Response™ Test (D29)

Fastpaque-Response™ test was done according to the manufacturer (Biotec Laboratories) instructions sheet**.**

3. Results

Epidemiology

Among the 90 patients, 54 (60%) were found to be males, while 36 (40%) were female. Out of the 90 patients, 18 (20%) were classified as old cases depending on WHO criteria while 72 (80%) were considered as new cases. All ages were found to be affected with tuberculosis, the highest frequency was found among the age group 21 -50 years who were 60 (66.7 %), followed by age group >50 years who were 18 (20 %) while the lowest frequency was age group <20 years who were 12 (13.3%)

Bacteriological Findings

Direct ZN staining was performed to all samples (90), and were all found acid –fast bacilli positive**.** All other biochemical tests confirmed that out of 75 successfully cultured isolates, 60 isolates were members of MTC.

Mycobacteriophage Testing (D29)

Direct D29 Test (From Sputum)

Direct D29 test (FAST Plaque-Response™) was performed to the entire sputum specimens for the detection of rifampicin resistance. 70/90 (77.8%) gave

interpretable results, 9/90 (10%) gave none interpretable results and 11/90 (12.2%) reflected contamination. Out of the 70 interpretable results, 56 (80%) were rifampicin susceptible whereas 14 (20%) were rifampicin resistant as shown in Figure (1).

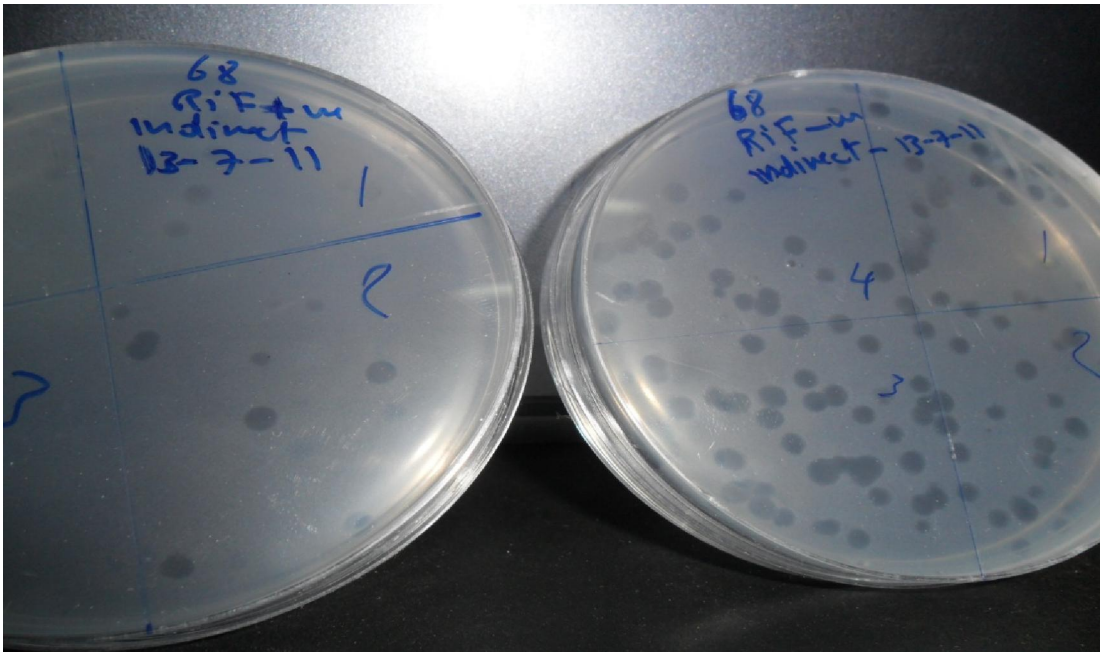

Figure 1. Direct D29 test of rifampicin susceptibility; Left: less than 50 plaques→Rif susceptible . Right: more than 100 plaques → Rif resistant

Indirect D29 Test (Growth Suspension)

The not interpretable and contaminated specimens (20) were retested with indirect D29 method. 12/20 (60%) were rifampicin susceptible (Figure 2), 7/20 (35%) were rifampicin resistant, whereas, 1/20 (5%) showed contamination.

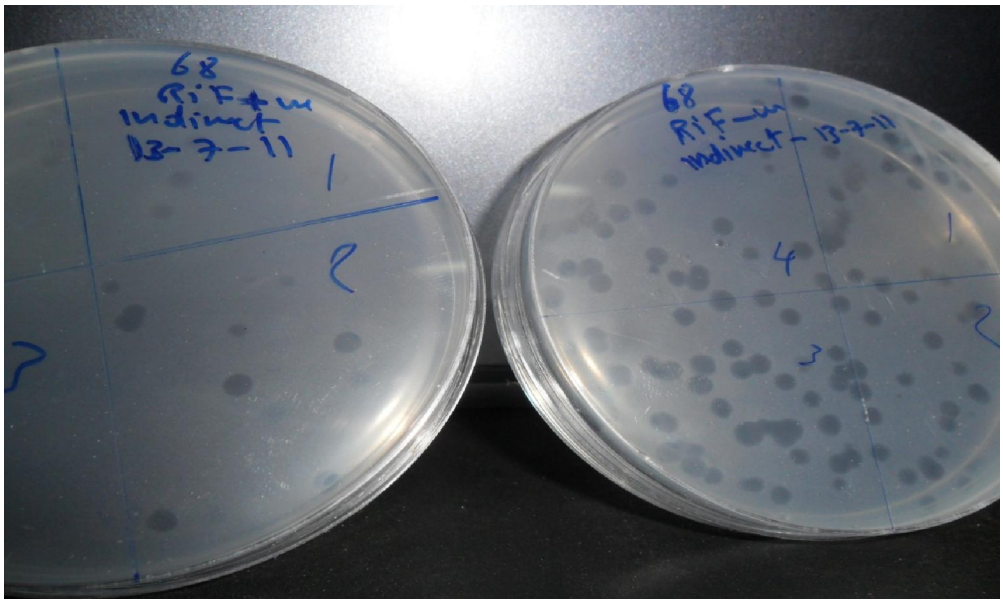

Figure 2. Indirect D29 test of rifampicin susceptibility; Left: less than 50 plaques → Rif susceptible. Right: more than 100 plaques → Rif resistant.

Polymerase Chain Reaction

Amplification of *IS6110*

From the seventy five *Mycobacterium tuberculosis* complex isolates which showed rapid or slow growth, 65 (86.7%) were subjected to PCR. 60/65 (92.3%) isolates showed a band typical in size (123 bp) to the target gene (*IS 6110*) as indicated by the standard DNA marker, the remaining 5 (7.7%) isolates were negative, as shown in Figures 3.

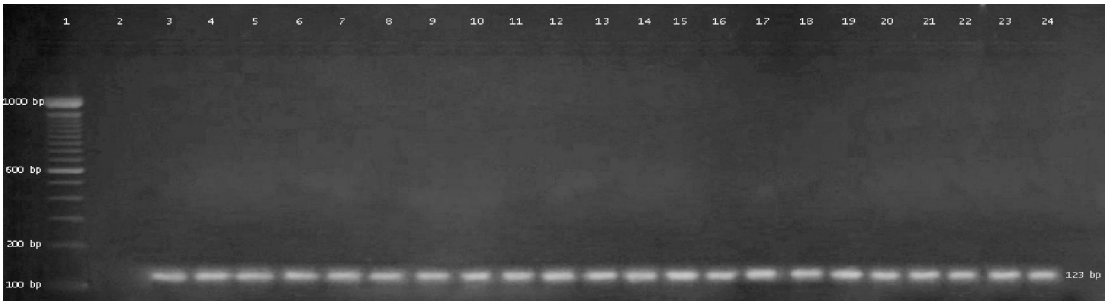

Figure 3. The amplicon of *IS 6110* gene (123 bp) on 1.5% agrose gel stained with ethidium bromide; lane 1 DNA marker, lane 2 negative control, lanes (3-23) patient's samples showing positive results for MTC (123 bp), lane 24 positive control.

Detection of rpoB Gene

All the 60 PCR positive isolates were subjected to rpoB gene amplification by PCR. 15/60 (25%) gave band typical in size to the target rpo B gene (193 bp) as indicated by standard DNA ladder for the presence of rifampicin resistance (Figure 4).

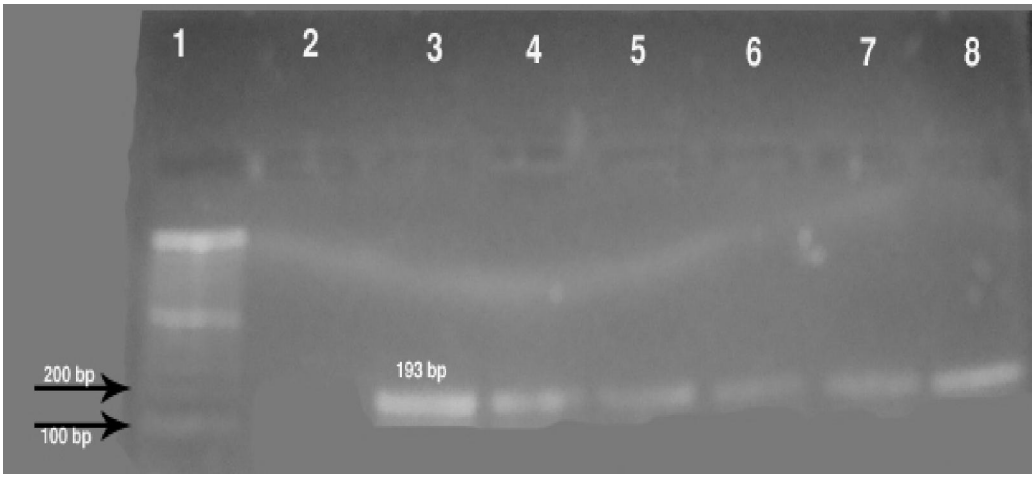

Figure 4. The amplicon of rpoB gene (193 bp) on 1% agrose gel stained with ethidium bromide. Lane 1 DNA marker, lane 2 negative control, lanes (3-7) patient's samples showing positive results for rpoB rifampicin resistance, lane 8 positive control.

Correlation between PM, D29 and rpoB Gene

The correlation between the PM, D29 and rpoB gene reflected a significant variation between these three methods as shown in table 1.

Table 1. Number of rifampicin resistant strains by proportion method (PM), phage and rpoB

Rifampicin	PM		Phage		rpoB Gene	
	No.	%	No.	%	No.	%
Resistant	18	30	21	35	15	25
Sensitive	42	70	39	65	45	75
Total	60	100	60	100	60	100

4. Discussion

MDR and XDR tuberculosis are serious threats to the progress that has been made in the control of tuberculosis worldwide over the past decade [3]. An estimated 4.8% of all new and previously treated TB cases diagnosed worldwide in 2006 were MDR TB [4]. Because patients with MDR TB are resistant to treatment with first-line drugs, they could be treated with second-line drugs that are more expensive, have more side effects, often require injection, and involve longer treatment.

Conventional methods for susceptibility testing require several months before results can be reported. However, rapid methods to determine drug susceptibility have been developed. Phage assay have been reported as a rapid useful tool for antimicrobial susceptibility testing [5].
The aim of the present study was to evaluate the performance of mycobacteriophage D29 technology in detecting rifampicin resistant tuberculosis directly from smear positive sputum. This was compared with proportional and molecular method through amplification of rpoB gene. Also it aimed to estimate the prevalence of MDR TB in Kassala State.

Phage assay results are available in 2-3 days; whereas the proportion method results are obtained within 63-70 days from receiving sputum samples [13]. The sensitivity of the phage assay in detecting RMP resistance in this study was 100 % while the specificity achieved was 93.3 %. In a meta-analysis done by Jessica and Madhukar (2009) [14] for 15 studies for commercial phage amplification assays, the sensitivity ranged between 81 and 100%, and specificity between73 and 100%.

The results of the present study agree with those of Kisa *et al.* (2003) [15] who studied 88 isolates in Turkey using mycobacteriophage D29, he recorded 100% sensitivity and 92.5% specificity. Albert *et al.* (2004) [16] in Cape Town, South Africa, achieved a sensitivity of 100% and a specificity of 100%. Richard *et al.* (2007) [17] obtained a sensitivity of 98.1% and a specificity of 96.3% in South Africa. These results are different from those of Butt *et al.* (2007) [18] in Pakistan who found a lower sensitivity and specificity to D29 phage assay which were 86% and 73% respectively. The negative predictive value of D29 phage test in the present study was 100%. This means that the test can potentially be used to rule out rifampicin resistance while it has a relatively lower positive predictive value of 85.7%. This implies that when it is positive it needs to be further confirmed by another standard test like DST.

Drug susceptibility testing applied in the present study revealed that 19/60 (31.7%) were resistant to rifampicin, 20/60 (33.3%) were resistant to isoniazid, 18 (30%) were resistant to both rifampicin and isoniazid (MDR). This is consistent with the results of Khalid (2009) [19] who studied the characterization of drug resistance in Kassala State and found 23/ 53 resistant isolates, among which rifampicin had the highest number of resistance (13/23) but the multi-drug resistance was detected in 5 isolates only. Rifampicin resistance can be assumed to be a surrogate marker for MDR TB, since more than 90% of rifampicin isolates are also isoniazid resistant [20]. The combined resistance to rifampicin and isoniazid observed in this study was 90%. This result agrees with that observed by Elhassan *et al.* (2012) [21] who studied 56 strains in Sudan and found that 32/56 of the strains were resistant to RIF, 36/56 to INH and 30/56 were resistant to both drugs (MDR) calculated with a combined resistance of 83%. Contamination rate to D29 results in this study was found 12.2 % although the sputum samples were decontaminated and nystatin-oxacillin-amikacin (NOA) was added to reduce overgrowth of contaminating micro-organisms present in the sputum. Similar rate of contamination was also encountered by Albert *et al*., (2007) [22] who recorded 14% contamination rate, But these rates were much higher compared to the rate observed by Richard *et al.,* (2007) [17] who was able to decrease the contamination rate to 0.8% although they used the same combination.

The contamination rate in DST method was 20% which is higher than the contamination rate in D29 method. The DST is also time consuming because the test needs to be repeated and to wait for another 42 days to get the final results.

PCR assay was used to characterize 65 out of the 80 examined isolates as *M. tuberculosis* complex. The sensitivity of the PCR reached 92.3% compared with the growth on LJ medium 80/90 (88.9%), 60/65 (92.3%) of the samples gave positive results (typical band at 123 bp) and 5 (7.7%) were negative (no bands). These findings are in line with Tonjum *et al.* (1996) [23] who obtained 87.5% sensitivity.

In this study rpoB gene mutation was identified in 15 out of 18 rifampicin resistant strains with a sensitivity of 83.3%. This finding agrees with Xiao *et al.* (2003) [24] who analyzed a total of 39 rifampicin resistant *M. tuberculosis* isolates in Shanghai by this assay. They found 36 strains having mutations in rpoB gene resulting in 92.3% sensitivity. The presence of some isolates in this study, which were resistant by conventional test and appeared as sensitive by PCR method (rpoB gene), (3/18 (16.7%)), this is likely due to the fact that over 70 distinct rpoB mutations and four frequent mutations (codons 526, 513, 531 and 516) have been reported for RIF resistant *M. tuberculosis* isolates worldwide [25]. That means the possibility of the presence of other types of mutations that needs other primers and PCR protocol [21]. In addition to that, more than 95% of rpoB mutations in RIF's resistant clinical

isolates have been found within the rrifampicin resistance determining region –RRDR [26].

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