UJMR, Volume 6 Number 2, December, 2021, pp 19 - 23 *ISSN: 2616 - 0668*
https://doi.org/10.47430/ujmr.2162.003

Received: 7th August, 2021 **Accepted:** 15th September, 2021

Comparative Studies of the Methods of Detecting Rifampicin Resistant *Mycobacterium tuberculosis* among Tuberculosis Patients Attending Aminu Kano Teaching Hospital, Kano

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Abstract
This study was conducted to compare the specificity and sensitivity of GeneXpert MTB/Rif and Lowenstein-Jensen (LJ) Proportion methods of detecting rifampicin-resistant *Mycobacterium tuberculosis* among Acid Fast Bacilli (AFB) positive patients attending the Directly Observed Treatment (DOT) Centre of Aminu Kano Teaching Hospital Kano. A total of 150 AFB positive samples were collected and processed according to the guideline given by National TB and Leprosy Control Program (2015) and WHO (2012), The result revealed that rifampicin-resistant *Mycobacterium tuberculosis* (RR-TB) from the samples was very high; 66.7% and 60.8% for GeneXpert MTB/Rif and Lowenstein-Jensen (LJ) Proportion methods respectively. Cohen's Kappa (interrater reliability) statistical analysis indicated a substantial agreement between GeneXpert and LJ Proportion specificity and sensitivity (Kappa value = 0.73).
Keywords: *Mycobacterium tuberculosis*, GeneXpert MTB/Rif, Lowenstein-Jensen (LJ) Proportion, Rifampicin resistance.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (MTB). Tuberculosis generally affects the lungs, but can also affect other parts of the body. Most infections do not have symptoms, known as latent tuberculosis. About 10% of latent infections progress to active disease which, if left untreated, kills about half of those infected. The classic symptoms of active TB are a chronic cough with blood, fever, night sweats, and weight loss (WHO, 2016).

There exist some test methods used in the detection of resistance in TB, among which WHO endorsed two; GeneXpert MTB/Rif assay and Lowenstein Jensen (LJ) Proportion Method. The GeneXpert MTB/Rif is an assay that detects the presence of TB and also Rifampicin resistant strain simultaneously. The phenotypic Lowenstein Jensen (LJ) Proportion Method (LJPM) is widely used in developing countries and is a proportional method that uses fixed concentrations of first-line TB drugs: isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and streptomycin (SM). This method is simple and cost-effective; however, the results require a long time to obtain and can be unreliable in some diagnostic examination settings (Agonafir *et al.*, 2010). The former method is reported in a research conducted in India to have 100% specificity in a large clinical GeneXpert validation study even though the specificity drops to 98.3% in a subsequent multicenter study (Van *et al.*, 2011). The use of the later method (LJPM) is largely embraced in our community. This study, therefore, evaluates the specificity and sensitivity of GeneXpert MTB/Rif assay and Lowenstein Jensen (LJ) Proportion Method (LJPM) in the detection of RR-TB.

MATERIALS AND METHODS

Sample Collection and Processing:

One hundred and fifty (150) screened and confirmed sputum samples from AFB positive patients attending Directly Observed Treatment (DOT) Centre of Aminu Kano Teaching hospital Kano, were collected and processed as described by the National TB and Leprosy Control program SOP NTBLCP (2015) and WHO (2012); two spots of sputum samples were collected from each patient at least one hour apart in a sterile leak-proof (50ml) wide mouth, screw-capped, leak-proof falcon tube container. The patients were instructed to rinse their mouth with water and take 3 to 4 deep breaths, holding the breath for 3-5 seconds after each inhalation and then cough after the last inhalation, emptying the sputum into the falcon tube with care not to contaminate the outside of the tube. The falcon tube screw cap was then closed tightly and wiped with cotton wool soaked in tuberculocidal disinfectant (Lysol). 5 ml of the specimen was collected. The sputum specimens were collected in an open and well-ventilated space, the positive AFB samples were confirmed by microscopy and then later stored at a temperature of 8^{o}C for further laboratory analysis which involves genotypic detection of *Mycobacterium tuberculosis* using GeneXpert MTB/Rif assay and phenotypic detection using culture (LJPM) method.

a. Genotypic Detection of *Mycobacterium tuberculosis* Using GeneXpert Assay

GeneXpert MTB/Rif (Cepheids, USA) was used to detect the presence of *Mycobacterium tuberculosis* and also Rifampicin-resistance, the procedure involves disinfection of the working area then the GeneXpert MTB/Rif cartridge was labelled with the sample number. The lid of the sputum specimen container was unscrewed and 2 volumes of sample reagent (SR) were added directly into 1 volume of sputum (ratio 2:1) and the lids were closed. It was then vortex and incubated at room temperature for 10 minutes. After 10 minutes of incubation, it was vortex again several times and incubated for 5 minutes. After an additional 5 minutes of incubation, the sample was perfectly fluids before being tested, with no visible clumps of sputum. But if the sample is still viscous, then another 5-10 minutes was added before inoculating into the cartridge. To inoculate the cartridge, a sterile pipette was used to transfer 2 ml of the liquefied sample into an open port of the GeneXpert MTB/Rif cartridge and closed immediately. The pipette was discarded into a tuberculocidal disinfectant solution for decontamination (NTBLCP, 2015).

Running of the Test

The tab, "Create a test" was clicked on a computer. A window requesting to scan the cartridge barcode appeared on the computer screen, a barcode scanner was used to scan the cartridge barcode and a window that requested patient's Names and Laboratory Serial numbers was opened and filled. After this was done, the "start" icon was clicked and so the assigned module, as indicated by the blinking of green light. The cartridge bay door of the selected module was opened and the cartridge was loaded carefully. By closing the cartridge bay door, it automatically started running the test (NTBLCP, 2015).

b. Phenotypic Detection of *Mycobacterium tuberculosis* using Culture

The collected sputum samples were processed before inoculation on growth media which involves three steps; digestion (to loosen mucoid material in sputum samples), decontamination (to limit contamination of cultures by fast-growing non-mycobacterial commensal organisms), and concentration (to increase the sensitivity of culture) (NTBLCP, 2011).

Preparation of Media and Reagent

The Lowenstein-Jensen (LJ) medium was used to isolate Mycobacteria; and was prepared as follows:

Preparation of Homogenized eggs

Eggs were prepared and homogenized for incorporation into LJ medium as described by NTBLCP SOP (2011) and WHO (2011**).** About 100 ml of liquid soap solution was mixed with 8 litres of water to get an alkaline solution. One day fresh eggs were placed in the alkaline soap solution for 30minutes. The eggs were scrubbed thoroughly and rinsed under running tap water. They were then soaked in 70% alcohol for 15 minutes and placed on sterile dishes to air dry the excess ethanol. The eggs were then cracked carefully into a sterile measuring cylinder until desired quantity was attained, homogenized using a sterile blender, and filtered using sterile gauze.

Preparation and Constitution of LJ medium

The Lowenstein Jensen (LJ) medium was prepared according to the manufacturer's recommendations. Exactly 37.2 g of the powder was suspended in 600 ml of purified water containing 12 ml glycerol. The suspensions were mixed thoroughly, heated with frequent agitation, and boiled for 1 minute to completely dissolve the powder. It was then autoclaved at 121ºC for 15 minutes and cooled to 45ºC - 60ºC. One litre of the freshly homogenized egg was added to the autoclaved mixture, mixed well and dispensed into sterile tubes, and allowed to coagulate in a slanting position at 85ºC for 45 minutes in an inspissator.

Isolation

The decontaminated-digested sediments of these sputum samples were then inoculated into the prepared LJ media in duplicates for each sample and then incubated aerobically at 37ºC for 8 weeks. The tubes were observed daily for the first week of incubation and weekly thereafter till eight weeks. Caps were opened once a week for a short interval to aerate the cultures and to examine tubes for positive growth cultures showing evidence of growth at any time during this period for typical non pigmented, rough, dry colonies on Lowenstein-Jensen medium, coupled with the AFB positive microscopy result, suggestive of *Mycobacterium tuberculosis* complex (NTBLCP, 2011 and Gambo *et al.,* 2013).

Drug Susceptibility Testing using Lowenstein Jensen (LJ) Proportion Method

Susceptibility testing of the MTBC isolates to Rifampicin (40 µg/ml) was determined by the proportion method using Lowenstein Jensen egg-based slopes as described by NTBLCP (2011). For each sample 10^{-1} of the MTBC was inoculated on the drug-containing medium of the tubes. Three drug-free LJ slopes were inoculated with 10^{-1}, 10^{-2}, 10^{-3} diluted suspensions of a 1.0 McFarland standardized Inoculum. Furthermore, the rifampicin-susceptible MTB reference strain ATCC 27294 (H37Rv) was used as a susceptible control and known resistant strains (ATCC35838 H37Rv for RMP) was used as resistant controls. The slopes were incubated at 37°C and read after 4 and 6 weeks.

Reading of Results

After 28 days of incubation, slopes were observed for growth. The average number of colonies obtained from drug-containing slopes indicated the number of resistant bacilli contained in the inoculum. Dividing the number of colonies in the drug-containing slopes by those in the drug-free slopes gave the proportion of resistant bacilli. An isolate was considered resistant if the proportion of bacilli resistant to the critical concentration of the drug exceeded 1%.

RESULTS

The result from the Genotypic detection of *Mycobacterium tuberculosis* showed that out of the AFB positive samples earlier confirmed by microscopy, *Mycobacterium tuberculosis* was detected in all the samples (100%), culture revealed that 120 (80%) were positive as shown in Table 1.

Table 1: Detection of *Mycobacterium tuberculosis* by Genotypic and Cultural Methods

Type of Assay	No. Positive for *M. tuberculosis*	Percentage (%)
Genotypic	150	100
Cultural	120	80

Table 2 shows the susceptibility of *M. tuberculosis* to Rifampicin. Out of 150 *M. tuberculosis* detected genotypically, 100 (66.7%) were resistant to rifampicin, while 73 (60.8%) out of the 120 isolates detected by culture were found to be rifampicin resistant.

Table 2: Susceptibility to Rifampicin Based on Cultural and Genotypic Methods

Method	No. Screened	No. Sensitive (%)	No. Resistant (%)
Phenotypic (LJPM)	120	47 (39.2)	73 (60.8)
Genotypic (GeneXpert)	150	50 (33.3)	100 (66.7)

The specificity and sensitivity of GeneXpert MTB/Rif (genotypic) and Cultural methods of detecting rifampicin resistance by Kappa analysis TB are presented in Table 3. The result indicated a substantial agreement between GeneXpert and culture.

Table 3: Comparison of Specificity and Sensitivity of Genotypic and Culture Methods

Culture	Gene Xpert		Total
	Rif Resistant	**Rif Sensitive**	
Resistant	71	2	73
Sensitive	13	34	47
Total	**84**	**36**	**121**

Kappa value = 0.73

Interpretation:

K = 0.73 (0.61 to 0.81) indicated a substantial agreement between GeneXpert and culture results.

DISCUSSION

This study reported 100% conformity of the GeneXpert MTB/Rif TB detection method with that of the microscopy, as all (100%) of the AFB positive microscopy samples were also confirmed positive by the GeneXpert MTB/Rif genotypic method, hence evidencing the reliability and efficiency of both detection methods. Even though, the microscopy detection method had been reported to be far from being sensitive (Peter, 2018).

With regards to the rifampicin resistance determination, this study revealed that 66.7% and 60.8% of the isolates were resistant to rifampicin genotypically (GeneXpert MTB/Rif) and phenotypically (Lowenstein Jensen (LJ) Proportion) respectively. The rifampicin resistance prevalence documented in this work was higher as compared to the findings of Omisore *et al.*, (2018) and Peter (2018) who respectively reported the prevalence of 10% (in Lahore, Pakistan) and 14.7% (in the Bayelsa state of Nigeria) using the GeneXpert MTB/Rif. The differences observed may be due to variation in the sampling locations and other antibiotic resistance determining factors.

Moreso, the slight differences (of 5.9%) in the resistance prevalence of both methods suggest their reliabilities for resistance detection in MBT isolates, this conforms with the findings of Munir *et al.*, (2018) who made a comparison of Gene Xpert MTB/RIF Assays with Conventional Standard Proportion Method for Determination of Drug Susceptibility In Multidrug Resistant TB Suspects. The GeneXpert MTB/Rif method of detecting rifampicin-resistant TB had higher specificity and sensitivity than the conventional LJPM culture method, even though statistically was substantial agreement between GeneXpert MTB/Rif and Lowenstein Jensen (LJ) Proportion (Culture) method (Kappa value = 0.73). The high specificity and sensitivity of GeneXpert MTB/Rif may be because GeneXpert is based on genotypic-character detection.

Measures to curtail the aggravating factors (such as poor diagnosis, management and treatment) to the TB infections should be put in emplace. Tuberculosis (TB) infections and the rapid emergence of drug-resistant TB remain public health concerns in developing countries and even the globe, Nigeria is not an exception to this (Ugwu *et al.*, 2020).

CONCLUSION

The study evidenced that both the GeneXpert MTB/Rif (genotypic) and Lowenstein-Jensen (LJ) Proportion (phenotypic) methods are reliable and efficient. A concordance between genotypic and Phenotypic methods of detecting rifampicin-resistant TB was also observed (Kappa= 0.73), this suggests that both methods can be reliable in detecting rifampicin-resistant TB. However, the GeneXpert MTB/Rif susceptibility testing method has higher specificity and sensitivity.

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