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Performance Evaluation, Comparison and Degree of Effectiveness of Five Different Techniques for Detection and Isolation of Mycobacterium Tuberculosis

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ABSTRACT

This study was aimed to evaluate and compare the clinical performance and effectiveness of five different techniques for detection and isolation of Mycobacterium tuberculosis to determining the best technique for the detection and isolation of MTB at resource limited province of Pakistan, Balochistan. A cross-sectional retrospective cohort study on 307 samples collected from 2016 to 2018 was conducted at Fatima Jinnah General and Chest Hospital Quetta (FJHQ). All the samples were cultured according to the WHO protocol on LJ media and BACTEC 960 system. The fluorescence microscopy, ICT (MPT64 Kit) and ZN microscopy was used as confirmatory techniques. IBM-SPSS version 23 was used to analyze the recorded data and compute all the statistics. Multi Variate Binary Logistic Regression (MVBLR) analysis was used to determine the technique of choice best for the detection and isolation of MTB in clinical isolates. The frequency of positive samples processed by BACTEC 960, ICT (MPT64 Kit), ZN Microscopy, fluorescence Microscopy and LJ media culture were 39.7% (122/307), 38.1% (117/307), 39.4% (121/307), 39.1% (120/307) and 45.3% (139/307) respectively. The ICT (MPT64 Kit) and LJ media culture was found with lowest and highest recovery rate, sensitivity, specificity, PPV and NPV respectively. The overall comparative recovery rate was as follow ICT (MPT64 Kit) < fluorescence microscopy < ZN microscopy < BACTEC 960 < LJ media. The LJ media has highest recovery rate and reversal time with minimum contamination rate of 14/351 (3.98 %) as compared to BACTEC 960 system having the highest contamination rate of 46/351 (13.10 %) and exception of analyzing blood and urine samples. ICT (MPT64 Kit), fluorescence microscopy and ZN microscopy are found an essential confirmatory technique.

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1. Introduction

Tuberculosis (TB) is one of the major threats to the human health among infectious disease, affecting millions of people annually. Tuberculosis is the second leading cause of death, HIV being the first among the infectious disease. According to the 2013 estimates there were 9 million new TB cases while 1.5 (16.6%) million deaths. The increased number of Drug-resistant TB (DR-TB) mainly the multidrug resistant TB (MDR-TB) is the cause of increased number of deaths. According to the WHO report the frequency of MDR-TB in new cases and previously treated MDR-TB patient was 4% and 35% respectively, these percentages vary greatly indifferent countries (i.e. some countries may have >20% of MDR-TB among new cases while >50% among previously treated cases). Pakistan counts 5th among 22 high burden TB endemic countries and one of the major countries with highest TB mortality rate (WHO, 2013; WHO, 2014; Sinha & Mishra, 2015). One of the major causes of high mortality is the improper and false diagnosis and treatment of TB, the high TB mortality can be prevented by the access of the public to the proper and correct diagnosis and treatment techniques.

Tuberculosis being one of the utmost miserable infectious diseases requires certain conditions to be fulfilled by the body of infected individual (like immunocompromised or weakened immune system of the patient) these conditions are provided either by the comorbid health state (like HIV-AIDS) or age of the patient (like weakened immune system due to developing young age or developed old age). The multiplication of MTB in an infected adult with competent immune system will be hindered by its immune system resulting in limitation of development of symptomatically active tuberculosis from latent TB state as a result few individuals develop TB among exposed population during any stage of their life (Ndungu et al., 2013).

The proper diagnosis of TB forms the base of appropriate and on time treatment of disease, resulting in reduced mortality rate and better quality of life of patients. The microscopic methods like acid fast ZN smear (done by the compound microscope) and auramine-phenol staining (done by the LED-fluorescence microscope) remains one of the first-choice diagnostic tools for the detection of MTB in developing countries. The applications of these old aged techniques rather than latest more sophisticated and more sensitive techniques (like MGIT BACTEC 960, ICT MPT64 TB Ag Kit) are also considered one of the prime reasons of TB development rather than eradication in developing countries. In addition, LJ solid media culture and latest molecular based techniques with efficient result timing (like MTB/DR plus assay and Xpert MTB/RIF assay) are also used as diagnostic tools, but mostly used LJ solid media culture technique is time consuming (requiring about 6-8 weeks), while molecular techniques require expertise which are limited in poorly resourced and high burden TB countries like Pakistan (Kent & Kubika, 1985; Cheng et al, 2005; Negi et al., 2005).

The BACTEC 960 system containing liquid media (middlebrook 7H9) in specialized tubes called Mycobacterium Growth Indicator Tubes (MGIT) designed by Becton Dickinson (BD) Company is the latest non-radiometric technique introduced for the better recovery of mycobacterium (Lee et al., 2003). This technique is more sophisticated with capability of incubating 960 samples at a time and fully automated with self-generated result

reporting system (Abdel-Aziz et al., 2009). The BACTEC 960 system is designed to decrease the time and recovery rate than the LJ solid media culture (Lu et al., 2002). The BACTEC 960 system is placed in Provincial Reference Laboratory (PRL), Fatima Jinnah general and chest Hospital Quetta (FJHQ) and is run along the other techniques like ICT MPT64 TB Ag Kit, LJ media culture, ZN microscopy and LED fluorescence microscopy. The overall performance of BACTEC 960 system along with ICT MPT64 TB Ag Kit is unknown. Consequently, this study was aimed to evaluate and compare the clinical performance, effectiveness and need for more sophisticated programmed new techniques like Mycobacterium Growth Indicator Tube BACTEC 960 system, ICT (SD MPT64 TB Ag Kit), Ziehl-Neelsen (ZN) and LED Fluorescence Microscopy with gold standard manually operated Lowenstein–Jensen (LJ) solid media culture technique for detection and isolation of Mycobacterium tuberculosis. BACTEC 960 was comparatively assessed for its unique capability of supporting growth of all strains of mycobacterium that causes tuberculosis and reliance of patient for proper diagnosis.

The major benefit of BACTEC 960 system is that it has greatly reduced the turnaround time as compared to LJ media culture, but the significant drawback of BACTEC 960 system is that it is unable to differentiate between mycobacterium tuberculosis (MTB) from mycobacterium other than tuberculosis (MOTT), MGIT simply shows positive result for any growth in it, the growth may be of normal bacterial microbiota of the mouth present in the sputum or the fungal microbiota (candida albican) of the mouth. To inhibit the growth of microbes other than MTB a combination of five antibiotics called PANTA is used but the resistance to PANTA results in contamination and false result. The BACTEC 960 system being a sensitive technique still needs confirmation for MTB strains in positive cases. This confirmation may be done by the biochemical methods which are time consuming, laborious and biosafety precautious. The need for a better, less laborious and safe confirmatory technique is fulfilled with the discovery of a 28 kilo Dalton (kDa) antigenic structure specific for the MTB only (Hasegawa et al., 2002). Studies have shown that the recombinant as well as native MPT64 can distinguish between BCG vaccinated individuals and MTB infection (Oettinger et al., 1997). The Standard Diagnostics, Seoul, South Korea has manufactured a rapid, highly sensitive and specific serological immunochromatographic test (ICT) called SD TB Ag MPT 64 with the help of monoclonal antibodies used against MPT64 antigenic structure of MTB for the confirmation of MTB isolates in positive samples of MGIT/BACTEC 960 system and LJ solid media culture (Cheng et al., 2005; Negi et al., 2005).

As the microscopy is no more considered standard for MTB diagnostic purposes while the LJ media culture is time consuming and the MGIT/BACTEC 960 system is standard and considered one of the best methods with short turnaround time and a large capacity of incubating 960 samples at a time but is of no use without the ICT (SD TB Ag MPT 64 kit) to confirm the presence of MTB complex in positive samples of MGIT/BACTEC 960 system. In this study we mainly assess the sensitivity, specificity, diagnostic usefulness, and various statistical predictive values of the ICT (SD TB Ag MPT 64 kit) along with time consuming gold standard LJ media culture, MGIT/BACTEC 960 system, ZN and LED fluorescence microscopic techniques.

2. Methodology

2.1. Study Duration, Design and Settings

A cross-sectional retrospective cohort study was conducted at biosafety level 2 (BSL-2) microbiological Provincial Reference Laboratory (PRL) of Fatima Jinnah general and chest Hospital Quetta (FJHQ) Pakistan, the one and only provincial reference TB treatment facility at biggest province of Pakistan, Balochistan from February 1st, 2016 to September 25th, 2018. All the samples received from the patients were evaluated with five different techniques (LJ solid media culture, MGIT/BACTEC 960 system, SD TB Ag MPT 64 ICT kit, ZN and LED fluorescence microscopy) These patients were received at FJHQ belonging from various districts of Balochistan, covering patients from almost all the districts of the province as well as the foreigner patients received from western and southern provinces of Afghanistan. Balochistan is one of the high burden TB provinces of Pakistan and almost more than 90% of DR-TB patients from Balochistan as well as western and southern Afghanistan are treated at FJHQ.

2.2. Study Population and Sample Description

Pakistan to be processed by the LJ solid media culture and MGIT/BACTEC 960 system to diagnose the MTB complex in these patients. LJ solid media culture is accompanied by the LED Fluorescence microscopy while MGIT/BACTEC 960 system is accompanied by the SD TB Ag MPT 64 ICT kit and ZN microscopy as per WHO protocol. Hence, a single sample sent to be processed by two techniques (LJ media culture and MGIT/BACTEC 960 system) is now processed by five techniques (LJ media culture, MGIT/BACTEC 960 system, ICT, ZN and LED fluorescence microscopy). A total of 307 out of 351 samples were selected for this study the rest of 44 samples were omitted either due to the contamination or unclear results for all the stated five techniques.

2.3. Inclusion Criteria

All samples with clear (either positive or negative) outcome for all the techniques are included in this study. The samples were either collected at the PRL or collected remotely by a healthcare provider sent to the PRL for the processing. The samples included Broncho-Alveolar Lavage (BAL), Cerebro-Spinal Fluid (CSF), Gastric Lavage (GL), Muco-Purulent Sputum (MPS), Pleural Fluid and Pus to be processed for the detection of MTB. A sample stored for < 2 days was included in this study.

2.4. Exclusion Criteria

The contaminated samples were excluded from this study. The samples containing large impurities which cannot be processed by all the techniques are excluded from this study. Tissue sample like lymph nodes were excluded from this study as lymph nodes cannot be processed by all five techniques. A sample stored for > 2 days was excluded from this study.

2.5. Laboratory Methodology

N-acetyl-l-cysteine and sodium hydroxide (NALC/NaOH) was used to process the sample and a centrifuge of about 3000 round per minute (RPM) for 15 minutes is used to concentrate the processed sample. The sediments were discarded while sterile buffer of phosphate is used to re-suspend the sediments to a volume of about 2ml. This 2ml suspension is used to be inoculated in LJ solid media culture slants (about 2-4 drops), middlebroke 7H9 liquid media of MGIT/BACTEC 960 system (0.5ml) and finally used to prepare an Acid-Fast Bacilli (AFB) staining smear. Auramine-phenol is used to stain the smear and examined by the LED fluorescence microscope. The sample collection and processing were done on the same day. Samples received at the weekends that cannot be processed on the same day were stored at 2-6 °C for < 2 days in case of delay the samples are discarded and a fresh sample is collected from the patient. All the processing techniques were done as per WHO protocol.

2.6. Culture of MTB on LJ Solid Media Culture

The received samples, after processing was inoculated into the LJ solid media culture slants (2-4 drops) as per WHO protocol. The sample was reported positive for LJ solid media culture upon the appearance of MTB colonies on the surface of LJ media slant. The LJ culture positive samples were finally confirmed by the AFB smear, stained by auramine-phenol and analyzed by the LED fluorescence microscopy. The LED fluorescence microscopy is not as such reliable tool as ICT (SD MPT64 TB Ag Kit) for the confirmation of positive LJ solid media culture. Unfortunately, the ICT (SD MPT64 TB Ag Kit) is not used for the confirmation of positive LJ solid media culture in microbiological PRL, FJHQ which is one of the limitations of our study. The LJ solid media slants were examined twice a week for the appearance of colonies on LJ slant surface. The LJ solid media slants were incubated for about 6-8 weeks at 37 °C until the MTB colonies have been appeared.

2.7. Culture of MTB in Middlebrook 7H9 Broth-Based Media in MGIT/BACTEC 960 System

The MGIT/BACTEC 960 system uses a specialized tubes called mycobacterium growth indicator tube (MGIT) containing 7 ml broth based liquid middlebrook 7H9 media. The media in MGIT contains Oleic acid, Albumin, Dextrose and Catalase (OADC) as nutritional substance for MTB and the combination of five antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) called PANTA in MGIT inhibits the growth of other microbes mainly the normal microbiota of the mouth present in the sputum samples and enables the growth of about all strains of mycobacterium.

The MGIT/BACTEC 960 system involves the inoculation of 0.5 ml processed sample into the MGIT and incubated at 37 °C for maximum 6 weeks (42 days). The tubes are automatically monitored for positive samples every 60 minutes. The MGIT contains a specific fluorescence sensor which is proportional to the amount of obligate aerobic microbes present. The sensor in MGIT detects the concentration of oxygen consumed by the bacteria in the culture tubes. The level of fluorescence is proportional to the consumption of oxygen by bacteria. A culture tube with a certain level of fluorescence is indicated positive by the MGIT/BACTEC 960 system. The positive samples were further confirmed by the ICT (SD MPT64 TB Ag Kit) and AFB smear stained by the acid-fast Ziehl-Neelsen (ZN) staining technique. The performance of MGIT/BACTEC 960 system without ICT (SD MPT64 TB Ag Kit) is not

reliable as any aerobic microbial growth in MGIT is reported positive by the MGIT/BACTEC 960 system, the ICT (SD MPT64 TB Ag Kit) is able to differentiate between mycobacterial and non-mycobacterial growth in culture tubes.

2.8. ICT (SD MPT64 TB Ag Kit) for the Confirmation of the MGIT/BACTEC 960 System

A rapid serological immunochromatographic test called ICT (SD MPT64 TB Ag Kit) is used to confirm all the samples reported positive by the MGIT/BACTEC 960 system. This technique works on the basis of antigen-antibody reaction. In this technique the MPT64 MTB protein functions as an antigenic structure for the antibodies fixed at the ICT kit. The ICT kit comprises of two invisible bands, a control band (C-band) determines the validity of the ICT kit and test band (T-band) determines the positivity and negativity of the test. 4-6 drops of broth based liquid middlebrook 7H9 media from the positive culture tube of MGIT/BACTEC 960 system is dropped into the sample well of the ICT kit. The appearance of C-band and T-band on an ICT kit indicates the validity and positivity of the sample respectively while the invisibility of C-band and T-band indicates the invalidity and negativity of the sample respectively.

2.9. Fluorescence Microscopy for the Confirmation of LJ Solid Media Culture

All the samples cultured in LJ solid media culture were also stained with acid-fast Auramine-Phenol staining technique. The stained slides were viewed under the 40x lens of Fluorescence Microscope. A sample is confirmed positive on the appearance of Greenish-Yellow colored Acid-Fast Bacilli under fluorescence microscope and the slides are kept for quality assurance. The Grading of Acid-Fast Bacilli is done according to the World Health Organization (WHO) and International Union Against Tuberculosis and Lung Diseases (IUATLD) Quantification scale for Auramine-Phenol staining as follow:

Table 1. Grading of smear in Fluorescence Microscope using 40x Objective Lens

Grading of smear in Fluorescence Microscope using 40x Objective Lens	
Number of AFB per Field or Length. 1 length = 200 HPF (High Power Field)	**IUATLD / WHO scale** (Grading / Result)
0 AFB / 1 Length	Negative (i.e. No AFB Detected)
1-19 AFB / 1 Length	Positive Scanty (Report Exact Figure)
20-199 AFB / 1 Length	1+ or (+)
5-50 AFB / 1 Field	2+ or (++)
>50 AFB / 1 Field	3+ or (+++)

The Auramine Phenol staining is more sensitive than ZN staining. The Mycolic Acid in cell wall of AFB retain the Auramine Phenol and appear greenish yellow in dark background of Methylene Blue after decolorizing the slide with Acid-Alcohol. All the remaining entities in the smear decolorizes with Acid-Alcohol and retains dark color of methylene blue.

2.10. ZN Microscopy for the Confirmation of BACTEC 960 System and ICT (MPT64 TB Ag Kit)

The positive MGIT/BACTEC 960 system samples were further confirmed by the ICT (MPT64 TB Ag Kit) and the Ziehl-Neelsen Carbol Fuchsin staining technique is used for confirmation of the ICT (MPT64 TB Ag Kit) result.

The ZN-stained slide is viewed under the Cedarwood Oil Emersion Lens on the bright field Compound Light Microscope. If red colored Acid-Fast Bacilli appeared in the slide, then it is reported as Positive and the slides are kept in slide boxes for quality assurance. The Grading is done according to the World Health Organization (WHO) and International Union Against Tuberculosis and Lung Diseases (IUATLD) Quantification scale for Ziehl-Neelsen staining as follow:

Table 2. Grading Acid-Fast Bacilli as per WHO and IUATLD recommendation

Grading Acid-Fast Bacilli as per WHO and IUATLD recommendation		
Number of Acid-Fast Bacilli	**Number of Oil Immersion Fields Examined**	**Grading/Reporting**
No AFB / 100 fields	100 Oil Immersion Fields	Negative (i.e. No AFB Detected)
1-9 AFB / 100 fields	100 Oil Immersion Fields	Positive Scanty (Report Exact Figure)
10-99 AFB / 100 fields	100 Oil Immersion Fields	1+ or (+)
1-10 AFB / Each field	50 Oil Immersion Fields	2+ or (++)
>10 AFB / Each field	20 Oil Immersion Fields	3+ or (+++)

The Mycolic Acid in the cell wall of Acid-Fast Bacteria gains red colored Ziehl-Neelsen Carbol Fuchsin stain and appears red in blue background (due to Methylene Blue) after decolorizing it with Acid-Alcohol (i.e. the Acid-Alcohol decolorizes all the entities not having mycolic acid as their integral part except for Acid-Fast organism).

2.11. Study Outcomes

The chief outcome of the samples in this study after being processed and analyzed by the five different diagnostic techniques was that ZN and fluorescence microscopy is only useful to some extent for the confirmatory purposes only and cannot be used solely for the detection of MTB in clinical isolates while the LJ solid media culture and MGIT/BACTEC 960 system are time consuming and needs secondary confirmatory test. The two widely used techniques are of no use without secondary confirmatory test. The outcome of this study concludes that the MGIT/BACTEC 960 system is reliable if accompanied by the ICT (SD MPT64 TB Ag Kit) and is less time consuming as compared to the LJ solid media culture. The outcomes also suggest that the ICT (SD MPT64 TB Ag Kit) should be used instead of fluorescence microscopy as a secondary confirmatory test for the LJ solid media culture. The ICT (SD MPT64 TB Ag Kit) has a rapid turnaround time of maximum 20 minutes and is the best choice for the confirmation of LJ solid media culture and MGIT/BACTEC 960 system instead of ZN or fluorescence microscopy due to its increased sensitivity and specificity.

2.12. Data Abstraction and Processing

Patients' data were retrospectively abstracted from Electronic Nominal Record System (ENRS), medical charts and Laboratory files. Most of the laboratory files and charts data were available in hard copies, saved in the archive of FJHQ. These hard files were transferred into the soft file so that easily be analyzed. All the personnel data of every individual patient were omitted so that to protect the confidentiality and privacy of the patients.

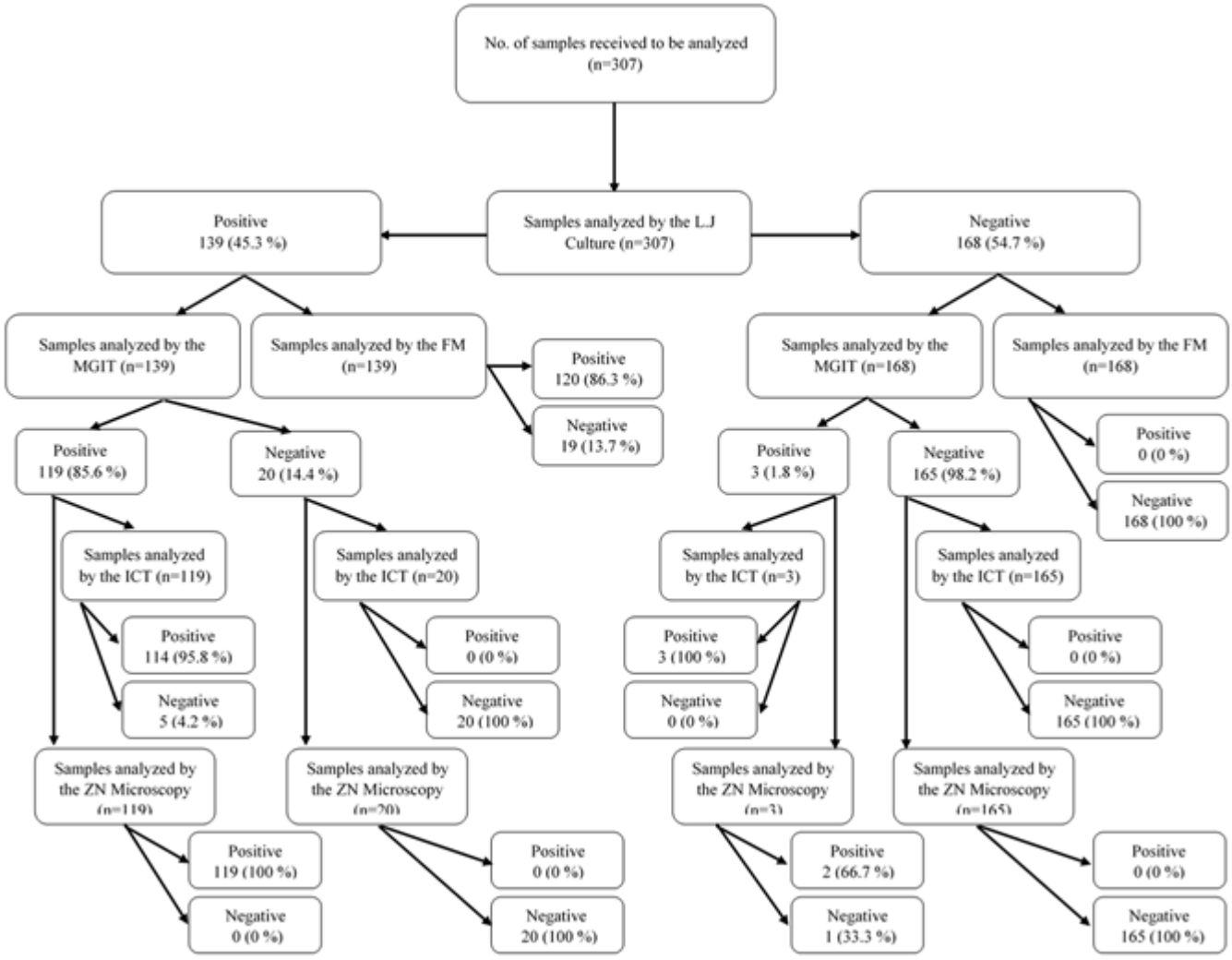
2.13. Data Analysis

Data were analyzed by SPSS version 23. Descriptive statistics were used to analyze the data. Continuous variables were compared through categorical variables and t-test using the Fisher exact or Chi-square test. Multi Variate Binary Logistic Regression (MVBLR) analysis was used to determine the technique of choice best for the detection

of MTB in clinical isolates. The inferential stats were used to comparatively study the outcomes of all five techniques as well as the validity and dependence of one technique without another particularly the validity of LJ solid media culture and MGIT/BACTEC 960 system without the ICT (SD MPT64 TB Ag Kit). A P-value of <0.05 was considered statically significant.

2.14. Ethical Statement

The medical ethical committee and authorities of Fatima Jinnah general and chest hospital Quetta (FJHQ) permitted this study. The focal person and head of pulmonology department FJHQ collaboratively agreed to this study. This study was approved by the faculty of pharmacy and health sciences, university of Balochistan (UoB) Quetta as well. A collaborative research agreement was made between the investigators UoB Quetta and collaborators and data provider authorities of FJHQ.



3. Results and Discussions

Out of 307 samples processed by MGIT/BACTEC 960 system, ICT (SD MPT64 TB Ag Kit), Ziehl-Neelsen (ZN) Microscopy, LED Fluorescence Microscopy and Lowenstein–Jensen (LJ) solid media culture technique, the frequency of positive samples reported and recorded from stated techniques were 39.7% (122/307), 38.1%

(117/307), 39.4% (121/307), 39.1% (120/307) and 45.3% (139/307) respectively. The ICT (MPT64 Kit) and LJ media culture was found with lowest and highest recovery rate, sensitivity, specificity, PPV and NPV respectively. The overall comparative recovery rate was as follow ICT (MPT64 Kit) < fluorescence microscopy < ZN microscopy < BACTEC 960 < LJ media. The reversal time for positive and negative samples of LJ media culture and BACTEC 960 was (16-31) days and (45-60) days respectively while the reversal time for ICT, ZN and fluorescence microscopy was instant.

Demography	Techniques used (Positive: P, Negative: N)									
	L.J Result		FM of L. J		MGIT Result		ICT of MGIT		ZN Microscopy of MGIT	
	P	N	P	N	P	N	P	N	P	N
Frequency (%)	139 (45.3)	168 (54.7)	120 (39.1)	187 (60.9)	122 (39.7)	185 (60.3)	117 (38.1)	190 (61.9)	121 (39.4)	186 (40.6)
Gender (n=307)										
Female (n=185)	97	88	85	100	86	99	83	102	85	100
Male (n=122)	42	80	35	87	36	86	34	88	36	86
History										
New patients	26	42	20	48	20	48	20	48	20	48
Previously treated	113	126	100	139	102	137	97	142	101	138
TB type										
Non MDR-TB	44	71	36	79	38	77	38	77	37	78
MDR-TB	95	97	84	108	84	108	79	113	84	108
Sample site										
Pulmonary	133	147	116	164	118	162	113	167	117	163
Extra-Pulmonary	6	21	4	23	4	23	4	23	4	23
Sample type										
BAL (Clear)	9	11	7	13	6	14	6	14	6	14
CSF	2	8	1	9	2	8	2	8	2	8
GL	2	5	2	5	2	5	2	5	2	5
Sputum (MP)	122	131	107	146	110	143	105	148	109	144
Pleural fluid	3	12	2	13	1	14	1	14	1	14
Pus	1	1	1	1	1	1	1	1	1	1
Age categories (Year)										
<10	3	10	2	11	3	10	3	10	3	10
10-20	17	21	13	25	16	22	16	22	16	22
21-30	32	49	31	50	27	54	26	55	27	54
31-40	31	33	27	37	30	34	27	37	30	34
41-50	12	18	11	19	12	18	12	18	11	19
51-60	27	18	21	24	18	27	17	28	18	27
61-70	13	15	13	15	14	14	14	14	14	14
>70	4	4	2	6	2	6	2	6	2	6

A total number of 351 samples were received and processed for the MTB detection, 12.5% (44/351) samples were reported as contaminated and the contamination rate for the MGIT/BACTEC 960 system and Lowenstein–Jensen (LJ) solid media culture was 12.5% (44/351) and 3.9% (14/351) respectively. The contamination rate for LJ solid media culture is comparatively low due to its secondary confirmatory test by the less sensitive LED Fluorescence Microscopy. The contamination rate for LJ solid media culture will rise if the secondary confirmatory test is done by the ICT (SD MPT64 TB Ag Kit) instead of LED Fluorescence Microscopy. The test result for ICT MPT64 kit, ZN and LED fluorescence microscopy is either positive or negative and does not contain any contamination.

Demography	Techniques used (Positive: P, Negative: N)									
	L.J Result		FM of L. J		MGIT Result		ICT of MGIT		ZN Microscopy of MGIT	
	True Condition		True Condition		True Condition		True Condition		True Condition	
	P	N	P	N	P	N	P	N	P	N
Screen Test Outcome (%)	139 (45.3)	168 (54.7)	120 (39.1)	187 (60.9)	122 (39.7)	185 (60.3)	117 (38.1)	190 (61.9)	121 (39.4)	186 (40.6)
Test Outcome (+ve)	TP=139	FP =0	TP=120	FP = 0	TP=119	FP = 3	TP=114	FP = 3	TP=119	FP = 2
Test Outcome (-ve)	FN = 0	TN=168	FN =19	TN=168	FN =20	TN=165	FN = 25	TN=165	FN = 20	TN =166
Condition (+ve & -ve) Matrix	P = 139	N =168	P = 139	N =168	P = 139	N = 168	P = 139	N = 168	P = 139	N = 168
Sensitivity (TPR)	1		0.86		0.85		0.82		0.85	
Specificity (TNR)	1		1		0.98		0.98		0.98	
Precision (PPV)	1		1		0.97		0.97		0.98	
NPV	1		0.89		0.89		0.86		0.89	
Miss Rate (FNR)	0		0.13		0.14		0.17		0.14	
Fall-Out (FPR)	0		0		0.02		0.01		0.01	
FDR	0		0		0.02		0.02		0.01	
FOR	0		0.11		0.11		0.13		0.1	
Accuracy (ACC)	1		0.93		0.92		0.9		0.92	
Prevalence	0.45		0.45		0.45		0.45		0.45	
Likelihood Ratio (+ve)	∞		∞		42.5		82		85	
Likelihood Ratio (-ve)	0		0.13		0.14		0.17		0.14	
DOR	∞		∞		303.5		482.3		607.1	

Sensitivity (TPR)	= Σ True positive / Σ Condition positive
Specificity (TNR)	= Σ True negative / Σ Condition negative
Precision (PPV)	= Σ True positive / Σ Predicted condition positive
Negative Predictive Value (NPV)	= Σ True negative / Σ Predicted condition negative
Miss Rate (FNR)	= Σ False negative / Σ Condition positive
Fall-Out (FPR)	= Σ False positive / Σ Condition negative
False Discovery Rate (FDR)	= Σ False positive / Σ Predicted condition positive
False Omission Rate (FOR)	= Σ False negative / Σ Predicted condition negative
Accuracy (ACC)	= Σ True positive + Σ True negative / Σ Total population
Prevalence	= Σ Condition positive / Σ Total population
Likelihood Ratio (+ve)	= TPR / FPR

Likelihood Ratio (-ve) = FNR / TNR
Diagnostic Odd Ratio (DOR) = LR (+ve) / LR (−ve)
Condition Positive (P) = TP + FN
Condition Negative (N) = FP + TN
TP (True Positive), FP (False Positive), TN (True Negative), FN (False Negative

The non-radiometric broth based middlebrook 7H9 liquid media culture by MGIT/BACTEC 960 system is rapid and has improved turnaround time for the detection of MTB than the egg-based LJ solid media culture, hence recognized and widely recommended by the WHO. The application of MGIT/BACTEC 960 system is limited in scarcely resourced countries like Pakistan. Therefore, the present study was evaluated to increase and recommend the applicability of the MGIT/BACTEC 960 system over the LJ solid media culture. The MGIT/BACTEC 960 system accompanied by the secondary confirmatory ICT (SD MPT64 TB Ag Kit) test is the best method for detecting viable MTB, having a better time of detection and recovery rate than any other method. The LJ solid media culture has an increased turnaround time of about 2 months and is unable to differentiate between mycobacterium tuberculosis (MTB) and mycobacterium other than tuberculosis (MOTT) while the ICT MPT64 kit is able to differentiate MTB from MOTT and other microbes, thus the ICT MPT64 kit is recommended over the acid-fast staining techniques as a secondary confirmatory test for the LJ solid media culture.

The MTB detection by the LJ solid media culture was 45.3% (139/307), out of 139 positive samples 120 (86.3 %) samples were confirmed by the LED fluorescence microscopy (FM). The MGIT/BACTEC 960 system detected 39.7% (122/307) samples positive, out of 122 positive sample 117 (95.9%) and 121 (99.1%) samples were confirmed by the ICT (SD MPT64 TB Ag Kit) and ZN microscopy respectively. The increased confirmatory percentage of the MGIT/BACTEC 960 system makes it the technique of choice for the detection of viable MTB. The increased percentage of MTB detection by the LJ solid media culture indicates either contamination or the growth of MOTT. The comparative decreased percentage of MTB detection by the MGIT/BACTEC 960 system may be due to the death of MTB resulted from low pH or high pH during processing of the samples. Some studies have reported 60-70% death of the MTB during processing of the specimen (Kent & Kubica, 1984; Isenberg, 2004; Siddiqi & Rüsch-Gerdes, 2006). The contamination rate for LJ solid media culture and MGIT/BACTEC 960 system was 14/351 (3.98 %) and 46/351 (13.10 %) respectively which was comparatively similar (3-5%) with pre-established studies for LJ solid media culture and higher (5-8%) for the MGIT/BACTEC 960 system (Siddiqi & Rüsch-Gerdes, 2006). Similar contamination rates of 12.9%, 13.6%, 15.1% and 17% for the MGIT/BACTEC 960 system was reported from Turkey, Spain, Taiwan and U.S.A respectively (Phyffer et al., 1997; Lee et al., 2003; Feyzioglu et al., 2014). The contamination rate from Germany, Iraq and Nigeria was 8.1%, 4.8% and 7% respectively MGIT/BACTEC 960 system (Hanna et al., 1999; Brittle et al., 2009; AL-Mazini Mo et al., 2010). The difference in contamination rates may be due to the problems in different sample storage conditions, laboratory rejection criteria, patient instructions, transportations and sample collection (Siddiqi & Rüsch-Gerdes, 2006).

The average time to detect (TTD) smear positive samples for MGIT/BACTEC 960 system and LJ solid media culture was 15 and 30 days respectively. Similar TTD results were reported from India (13.1 days for MGIT/BACTEC and 23.9 days for LJ media culture) and Yugoslavia (13.7 days for days for MGIT/BACTEC and

22.1 days for LJ media culture) (Sewell et al., 1993; Mirovic et al., 2002). Another similar study from Pakistan by Luqman Satti et al. reported a shorter time of 11.2 days for MGIT/BACTEC 960 system (Satti et al., 2010). Similar shorter TTD results from Turkey and Taiwan were reported for MGIT/BACTEC 960 (8.3 and 9.1 days) and LJ media culture (20.1 and 17.6 days) respectively (Lee et al., 2003; Feyzioglu, 2014).

The average TTD of 20 days and 36 days were reported for smear negative patients in our study, comparable results were reported in Italian and Malaysian studies for MGIT/LG (13.3/36, 13.2/35.3 and 20/33.1 days) (Tortoli et al., 1999; Piersimoni et al., 2001; Mohd et al., 2009). The low bacillary load and nature of patient categories provides the reason for discrepancies between studies (Siddiqi & Rüsch-Gerdes, 2006).

Tuberculosis (TB) is the 2nd leading cause of death among infectious disease (HIV being the first among the infectious disease) worldwide, as the prevalence of death from HIV-AIDS in Pakistan is considerably less hence TB is the 1st leading cause of death among infectious disease in Pakistan. The death rate from TB can be reduced in high burden TB countries like Pakistan by early diagnosis in Laboratories well equipped with MTB detection tools like MGIT/BACTEC 960 system, LJ solid media culture and DST. The early diagnosis of TB is vital in the management of the TB patients. The isolation of MTB has improved by the automated systems, but the rapid identification of these MTB isolates is still needed. The ICT (SD MPT64 TB Ag Kit) is found to be the ideal and technique of choice in the rapid identification of the MTB clinical isolates. The ICT (SD MPT64 TB Ag Kit) is economical, rapid and much more reliable than the modern molecular methods in poorly resourced laboratories in resource poor countries like Pakistan.

The primary objective of this study was to assess an economically feasible and rapid test which precisely identifies the MTB in clinical isolates cultured in MGIT/BACTEC 960 system and LJ solid media culture. The differential identification of MTB and MOTT is clinically important in management and treatment of disease. The MTB is identified by conventional biochemical test which needs special biosafety measures that are not available in resource poor settings. The immunological, molecular and microbiological studies of mycobacterium resulted in identification of various useful antigenic structures specific to the MTB, among them MPT64 also called protein Rv1980c secreted by the viable MTB is one such antigen (Yamaguchi et al., 1989; Oettinger & Andersen, 1994; Wang et al., 2007). The MPT64 antigenic structure is not secreted by Mycobacterium other than Tuberculosis (like Mycobacterium Leprae and Mycobacterium Bovis) and BCG strains. The cloning and sequencing of MPT64 gene confirmed the presence of MPT64 antigenic protein associated to MTB strains only (Gennaro, 2000). The sensitivity and specificity of ICT (SD MPT64 TB Ag Kit) was found to be 98.3% (302/307) and 100% (307/307) respectively. The specificity and sensitivity of the ICT (SD MPT64 TB Ag Kit) makes it the most useful tool for the detection and confirmation of the MTB in clinical isolates. Unlike the molecular or biochemical techniques, The ICT (SD MPT64 TB Ag Kit) does not need any specialized trained individuals or sophisticated equipment thus making it the best choice for secondary confirmatory test of the MGIT/BACTEC 960 system and LJ solid media culture.

4. Conclusions

The performance of MGIT/BACTEC 960 system accompanied by the secondary confirmatory test (ICT MPT64 Kit and ZN microscopy) is better than the LJ solid media culture for the isolation and detection of the MTB having shorter turnaround time for both smear positive and negative samples. However, efforts should be made to minimize the contamination rate in LJ solid media culture and MGIT/BACTEC 960 system. The application of ICT MPT64 TB Ag kit for the detection of MTB in clinical isolates examined by the BACTEC 960 system and LJ culture can be proved helpful in early detection and managing of the disease. The ICT MPT64 Kit is low-tech, rapid and simple with high sensitivity and specificity makes it the most useful diagnostic tool. Pakistan being 5th among high burden TB countries is in need of introducing ICT MPT64 Kit for the rapid diagnosis of MTB.

Abbreviation

FJHQ, Fathima Jinnah general and chest hospital Quetta; PRL, Provincial Reference Laboratory; MTB, mycobacterium tuberculosis; MGIT, Mycobacterium Growth Indicator Tube; ICT, Immunochromatographic test; ZN, Ziehl-Neelsen; LED, light emitting diode; FM, Fluorescence Microscopy; LJ, Lowenstein–Jensen; TTD, time to detect.

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Declaration of Competing Interest and Ethics

The absolute manuscript was read and approved by the author. The experiments were conceived, designed and performed by the author. Analysis of the data and paper writing was done by the author. Supervision was provided by the author. The author declares no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in OPS Journal belongs to the authors.

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