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ORIGINAL RESEARCH

Microbiological Investigations of Fine Needle Aspirates from Newly Suspected and Previously Treated Tubercular Lymphadenitis Patients

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Background: Extrapulmonary tuberculosis (EPTB), particularly tubercular lymphadenitis (TBLN), remains to pose a huge public health problem in Ethiopia. A significant number of TBLN patients who completed a full course anti-TB treatment regimen were reported to have enlarged lymph nodes and other TB-like clinical presentations. This could either be from a paradoxical reaction or microbiological relapse, possibly due to mono/multi-drug resistance.

Objective: To investigate the rate of mono and multidrug resistance patterns of *Mycobacterium tuberculosis* as a cause of the observed treatment failures in clinically diagnosed and anti-TB treatment (newly or previously)-initiated LN patients.

Methods: A cross-sectional study was conducted on 126 TBLN-suspected and previously treated patients between March and September 2022. Data were analyzed using SPSS (Version 26.0). Descriptive statistics were used to determine the frequency, percentage, sensitivity, specificity, and positive and negative predictive values. The level of agreement was determined using Cohen's kappa and a Chi-square test was used to measure the association between risk factors and laboratory test outcomes. A *P*-value <0.05 was considered statistically significant.

Results: *Mycobacterium tuberculosis* was confirmed in 28.6% (N=36) of the 126 cases using BACTEC MGIT 960 culture detection method. Approximately, 13% (N=16) of the samples were collected from previously treated TBLN patients, of which 5/16 (31.3%) were multi-drug resistant, 7/16 were drug-sensitive and 4/16 were culture negative. To rule out other non-tuberculous agents, all samples were grown on blood and Mycosel agar plates, and no growth was detected.

Conclusion: The emergence of drug resistant (DR) TB seems to not just be limited to pulmonary form but also to TBLN. In this study we observed a considerable number of microbiologically confirmed relapses among previously treated cases, possibly indicating the need for confirmation of drug resistance using rapid molecular methods or phenotypical methods during treatment follow up.

Keywords: tubercular lymphadenitis, relapse, drug sensitivity testing, DST, FNA

Introduction

Extrapulmonary tuberculosis (EPTB) accounts for 15–25% of the total tuberculosis (TB) cases reported globally, where tubercular lymphadenitis (TBLN) contributes to the majority of the cases.[1,2] TBLN patients who have completed a full course anti-TB treatment regimen have been shown to have enlarged lymph nodes and other TB-like clinical presentations in a significant number.[3] This could be because of a paradoxical reaction or microbiological relapse, possibly due to misuse of drugs for the treatment of the existing TB infection, causing the organism to be drug-resistant.[4–6]

Drug resistance (DR) to Rifampin (RR) and multidrug resistance (MDR) is a serious concern where the estimated proportion of MDR/RR among TB in Ethiopia in the year 2021 was 1.1% in new cases and 12% in previously treated

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cases.[7] A higher rate of DR (14%) was shown by another study from Ethiopia.[8] Another report from India has shown a higher rate of DR (17.4%) among previously treated cases.[9] The emergence of drug-resistant *Mycobacterium tuberculosis* (MTB) often results from a failure to respond to anti-TB drug treatment regimens.[10]

Of the many anti-TB drug susceptibility testing (DST) methods, the Mycobacterium growth indicator tube (MGIT) 960 system phenotypic DST is the most commonly used test to diagnose DR patterns of MTB[11] in different types of specimens, including fine needle aspirates (FNA), collected from TB patients. Molecular DST methods such as GenoType MTBDR*Plus* Line probe assay (LPA) and GeneXpert MTB/RIF assay are also available for the rapid and simultaneous detection of DR TB.[12,13] In resource-poor settings, such as Ethiopia, patients with TBLN and presenting with enlarged lymph nodes are often diagnosed clinically or using FNA cytology (FNAC) and if cytological findings are consistent with TB, patients will be put on anti-TB drugs without being screened for drug resistance. This empirical treatment approach will potentially expose patients unnecessarily to superfluous anti-TB drugs that could lead to emergence of mono or multidrug-resistant TB. Evaluation of DR in newly identified or previously treated TBLN suspected patients is critically important to understand the DR rate and pattern among these patients and contribute to the overall TB control and infection prevention strategy.

In Ethiopia, treatment of EPTB is mostly dictated by non-specific clinical presentation followed by pathological diagnosis; identification of drug resistance in EPTB patients is often not practiced and, hence, assessing the burden of drug resistance on EPTB patients is critically important for appropriate intervention.[14,15] This study aims to investigate the proportion of mono or multidrug resistant TB among newly-identified and previously treated patients with TBLN using phenotypic DST, LPA, and GeneXpert. In a parallel effort, we also aimed to investigate the presence of bacterial or fungal infections other than MTB as a cause of enlarged lymph node.

Methodology

Study Setting

A cross-sectional study was conducted on 126 newly suspected and previously treated TBLN patients attending ALERT Hospital (one of the tertiary referral hospitals in Addis Ababa, where TB patients with drug resistance and HIV are clinically managed) from March 2022 to September 2022, of which 110/126 (87.3%) were new cases and the remaining 16 (12.7%) were previously treated for TBLN patients. Participants were recruited from the TB ward and slightly larger proportions of them were females (77/126, 61.1%); in an age range of 1–80 years (with a median age of 25.5 years). Recruitment criteria involves patients presenting with enlarged lymph nodes with or without constitutional symptoms such as fever, fatigue, and weight loss, and having associated pulmonary tuberculosis.

Information on participants' existing and previous clinical treatment history and socio-demographic data were collected using structured questionnaires or chart abstraction from March 2022 to September 2022. All clinical and laboratory data obtained from the participants were entered into an excel spreadsheet which was then exported to SPSS (version 26.0, USA) and was used for statistical analysis. Descriptive statistics were used for determining the frequency and percentage. Sensitivity, specificity, and positive and negative predictive values (PPV & NPV, respectively) for DST methods were calculated by using BACTEC MGIT 960 as a gold standard. The level of agreement was determined using Cohen's kappa. To measure the association, Chi-square test was used and, in all instances, a *P*-value less than 0.05 was considered statistically significant.

The study received ethical approval, which complies with the Declaration of Helsinki, from the AHRI/ALERT Ethical Review Committee (AAERC) before its commencement; and written informed consent was obtained from all adult participants and/or parents/guardians of child participants before enrolment into the study.

Fine Needle Aspirate Cytology (FNAC)

The FNA procedure was performed by an experienced pathologist, where the samples were collected aseptically using 21-gauge needles from all suspected TBLN patients. A drop of FNA was placed on a microscope slide, air–dried, and stained with the Giemsa staining procedure.[16] Slides were then examined under a microscope by an experienced pathologist. Cytological examination of FNA smears was considered diagnostic of TBLN when the smears contained

a thick, yellowish material showing either necrotic background associated with the presence of lymphohistiocytic and the presence of a significant polymorphonuclear cell population or the presence of a granulomatous inflammatory reaction consisting of a giant cell and/or epithelioid cell clusters and lymphohistiocytic cell population.

Microbiological Procedures

The leftover FNA samples were further divided into two separate cryovials; one containing skim milk, Tryptone, Glucose, and Glycerine (STGG) transport media, and the other containing sterile PBS, where both were transported to AHRI TB and Bacteriology laboratory for mycobacterial, other bacterial, and fungal culture investigation.

The FNA sample was streaked onto blood agar plates; incubated aerobically in a 37°C incubator for 24 hours and examined for any visible growth macroscopically. A portion of the FNA sample was also inoculated on Mycosel agar for fungal identification and incubated aerobically at 37°C for 1 week. After incubation, the inoculated plate was examined for any visible growth macroscopically.

LJ and MGIT Tube Culture and MTB Isolate Identification

For MTB growth detection on Lowenstein-Jensen (LJ) containing or MGIT media, the FNA samples were subjected to standard 3% NaOH–2.9% Tri sodium citrate–0.5% NALC decontamination procedure, followed by pellet resuspension in 1 mL of PBS. Three to four drops of the resuspended samples were inoculated to either LJ media or MGIT tubes; incubated at 37°C and checked for contamination twice a week. Growth of MTB was then checked once a week for eight consecutive weeks before reporting negative results. The sample was considered negative if no visible growth was detected within 8 weeks of culture. A tube showing a growth pattern from LJ or having a positive signal from BACTEC MGIT 960 undergoes identification processes before recording it as positive.

A slide was prepared from the grown colony for acid-fast bacilli (AFB) Zeihl Nelson staining as previously described[17] for microscopic examination under a 100x oil immersion objective. In parallel, the FNA samples were diluted (1:3), incubated for 15 minutes and transferred into GeneXpert MTB/RIF cartridge (Cepheid, USA) for reading using Xpert machine. MTB growth was further characterized using the SD Bioline MPT64 antigen testing kit. Briefly, ~100 µL of the growth suspension either from the LJ culture plates or MGIT tube was placed and incubated in a kit well for ~ 15 minutes, where the presence of two-colored bands shows an indication of MTB growth.

Upon confirmation, grown MTB isolates were stored using a cryotube containing freezing media (Middle Brook 7H9 broth mixed with glycerol at a final concentration of 20%).

Drug Susceptibility Testing

BACTEC MGIT 960 System

The inoculation procedures were performed using the manufacturer's instructions. Briefly, grown isolates were inoculated on anti-TB drugs containing tubes, whose final concentrations were adjusted to 1.0 µg/mL for STR and RIF, 0.1 µg/mL for INH and 5 µg/mL for EMB. No antibiotic was added to the growth control tube. Following inoculation, the DST set carrier was entered into a BACTEC MGIT 960 in the order of the set carrier definition selected when performing the AST set entry feature. Reports were printed manually when the instrument flagged the DST set complete. The instrument generates a flag when the growth control reached a growth unit value of 400.

Line Probe Assay (LPA)

Grown MTB isolates were heat-killed on heat block at 95°C for 30 minutes followed by DNA extraction using a reagent provided with a Genotype MTBDR*Plus* kit (Hain life science, Germany) following the manufacturer's instructions. Extracted DNA was amplified using a biotinylated primer to amplify the drug resistance-determining gene. Hybridization of the labelled PCR product was then followed with specific oligonucleotide probes immobilized on the strip. Captured labelled hybrids were detected by colorimetric development enabling the detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. Second-line DST was performed using a Genotype MTBDRsl kit (Hain life science, Nehren, Germany).

Results

FNA Cytology, Molecular and Microbiological Testing

Among the entire study participants, FNAC detected 55/126 (43.6%) cases as positive for TB and 1/126 (0.7%) case as inconclusive, which was also detected as negative by both MGIT and LJ culture methods and GeneXpert. GeneXpert detected 42/126 (33.3%) cases as positive, of which four were resistant to RIF and one showed an indeterminate result even after repeat test. Both MGIT and LJ showed a negative outcome for an indeterminate result from GeneXpert, but it was shown to be positive by FNAC. The proportion of MGIT culture-detected TB was 28.6% (36/126). Both LJ and MGIT show a substantial agreement (Kappa value = 0.855) and a similar substantial agreement was also shown between GeneXpert and MGIT 960 (Kappa value = 0.852). (Table 1). All MTB isolates (n=36) from MGIT tubes detected were positive by AFB microscopy and by the MPT64 antigen test. There was no growth of common bacterial and fungal agents from blood and Mycosel agar plates. Supplementary File 1.

Phenotypic and Genotypic Drug Susceptibility Testing of FNA Samples

Among the MGIT-positive isolates, 5/36 (13.9%) were shown to be resistant isolates by BACTEC MGIT 960 DST test (Table 3), of which 4/5 (80%) were resistant to Isoniazid and Rifampicin and the other one was also resistant to Streptomycin. A similar drug resistance pattern was also detected using Genotype MTBDR*Plus* assay (5/36, 13.9%). The Genotype MTBDR*Plus* assay has shown a resistance pattern to Rifampicin to 4/5 (80%) of the entire resistant isolates and the other one was resistant to Isoniazid only. GeneXpert MTB/RIF showed a RIF resistance pattern to 4/36 (11.1%) from the entire MGIT-positive isolates. The overall sensitivity and specificity of MTBDR plus was 100%. The sensitivity and specificity of GeneXpert MTB/RIF were 80% and 100%, respectively. The kappa value of genotypic DST methods with BACTEC MGIT 960 was shown to be in perfect agreement (Table 2). Five of the drug resistance isolates were screened for second-line drugs using a Genotype MTBDR*sl* assay and no resistance pattern was observed.

Isoniazid and Rifampicin Resistance and Mutation Patterns Using Genotype MTBDRPlus Assay

As shown in Table 3, the number of gene mutations associated with resistance to RIF (rpoB) and INH (katG and inhA) are depicted, where in four of the five resistant isolates, either WT7 or WT8 was missed with the presence of MUT2A

Table 1 Detection of MTB from Newly Suspected and Previously Treated TBLN Cases Using LJ, GeneXpert MTB/RIF Assay, and FNAC as Compared to MGIT 960 System

Methods		MGIT 960		Total	*P* value	Kappa
		Positive	Negative			
LJ	Positive	29	0	29	<0.0001	0.855
	Negative	7	90	97		
GeneXpert	MTB detected/RIF resistance detected	4	0	4	<0.0001	0.852
	MTB detected/RIF resistance not detected	31	7	38		
	MTB not detected	1	82	83		
	Indeterminate	0	1	1		
FNAC	Positive	35	20	55	<0.0001	0.635
	Negative	1	69	70		
	Inconclusive	0	1	1		

Table 2 Performance of MTBDR Plus and GeneXpert MTB/RIF as Compared to BACTEC MGIT 690

Drugs	MTBDRplus	BACTEC MGIT 960		Sensitivity	Specificity	PPV	NPV	Kappa	*P* value
		Resistant	Sensitive						
INH	Resistant	5/36	0/36	100%	100%	100%	100%	1	<0.0001
	Sensitive	0/36	31/36						
RIF	Resistant	4/36	0/36	80%	100%	100%	96.90%	0.873	<0.0001
	Sensitive	1/36	31/36						
INH and RIF	Resistant	5/36	0	100%	100%	100%	100%	1	<0.0001
	Sensitive	0	31/36						
GeneXpert MTB/RIF									
RIF	Detected	4/36	0	80%	100%	100%	96.90%	0.873	<0.0001
	Not detected	1/36	31/36						

Table 3 Mutation Pattern of Drug-Resistant *M. tuberculosis* Isolates

Gene	Missed Wild Type	Mutation	Change in Amino Acid	Resistance Pattern	Frequency (n=5)
rpoB	WT7	MUT2A	H526Y	MDR	2
	WT8	MUT3	S531L	MDR	2
katG	katG	MUT1	S315T1	MDR	4
inhA	WT1	MUT1	C15T	Mono resistance	1

and MUT3. Among the total drug-resistant isolates, 4/5 (80%) had a failure of the katG WT band with the corresponding presence of the katG MUT1 band.

BACTEC MGIT 960 Culture and DST Result of Previously Treated Cases

The data extracted from the chart review has shown that 16/126 (12.7%) participants were on anti-TB drugs previously and revisited the clinic for the second time, of these 31.3% (5/16) developed either mono or multidrug resistance to anti-TB drugs. Seven of the sixteen (43.7%) were culture positive and sensitive to the anti-TB drugs with both phenotypic and genotypic methods and 4/16 (25%) were culture negative. Among the previously treated cases, 12/16 (75%) had a positive culture result by BACTEC MGIT 960, of which 10/12 (83.3%) completed their full course of treatment. Of the 10 participants, four of them were MDR and one discontinued treatment, which also showed mono resistance to INH by both phenotypic and genotypic DST methods (Table 4).

Association of Drug Resistance Patterns with Previously Treated Cases and Clinics Revisiting TBLN Patients

Among the entire study, 16/126 (12.7%) participants were previously treated cases who were revisiting the clinic once again with enlarged lymph nodes and other symptoms mimicking TB. From the total 16 participants who had been previously treated, 5/16 (31.2%) had shown a drug resistance pattern to one or more of the first-line anti-TB drugs. On the other hand, 7/16 (43.75) of the participants who had been previously treated showed a positive culture result and were drug sensitive. Participants with prior history of treatment and clinic visits also showed negative culture results (4/16; 25%) (Table 5).

Table 4 Culture and DST Result of Previously Treated Cases, ALERT Hospital, Addis Ababa, Ethiopia

Parameters	Results	Took Anti-TB Treatment and Yet Relapsed[16]		*P* value	Completed Course of Treatment[14]		*P* value
		Yes	No		Yes	Discontinued	
BACTEC MGIT 960 culture	Positive	12	24	<0.001	10	2	<0.001
	Negative	4	86		4	0	
BACTEC MGIT 960 DST	INH	2	0	0.001	0	1	0.001
	INH &RIF	2	0		3	0	
	INH, RIF & STR	1	0		1	0	
	Sensitive	7	24		6	1	
Genotype MTBDRPlus	INH	1	0	0.003	0	1	0.005
	INH &RIF	4	0		4	0	
	Sensitive	7	24		6	1	

Table 5 Association Between Previous Treatment and Visit History with the Outcome of Drug Resistance Pattern

		BACTEC MGIT 960					Total	X^2	*P* value
		Sensitive	INH Resistant	INH & RIF Resistant	INH, RIF, & STR Resistant	N/A			
Previously treated cases (n)	Yes	7	2	2	1	4	16	37.6	<0.001
	No	24	0	0	0	86	110		
Visit History (n)	First visit	24	0	0	0	86	110		
	Second visit	7	2	2	1	4	16		

Discussion

In Ethiopia and other developing countries elsewhere, the diagnosis of TBLN is solely dependent on Fine Needle Aspirate Cytology (FNAC). In this study, a total of 126 participants were enrolled where they comprise newly-suspected and previously treated TBLN cases. Among them, FNAC has shown a detection rate of 43.6%. A similar report has been shown by one study conducted in Ethiopia.[18] On the other hand, a high detection rate and diagnostic capacity of FNAC have been reported from some studies.[19,20] Despite its higher sensitivity in diagnosing TBLN, several studies, on the contrary, have shown its limitation.[21] One of the commonly indicated limitations associated with FNAC as a diagnostic tool is that it mainly relies on finding the suggestive features of TBLN rather than finding the causative bacteria itself.[22]

According to some reports, TBLN patients who have finished their full anti-TB regimen came to visit the clinic once again with enlarged lymph nodes and symptoms consistent with TB. The presence of an enlarged lymph node after finishing the treatment course oftentimes is believed to be due to paradoxical reaction. Of note, there are also situations that such enlarged lymph node be caused by microbiological TBLN relapse.[5,23–26] Our study showed that, among the entire enrolled participants, 16/126 (12.7%) were found to be previously treated cases. A slightly similar finding has been reported by one study, with 15.6% of patients with enlarged lymph nodes post treatment.[27]

In this study, a statistically significant association was observed between the development of drug resistance and previously treated cases. Among the previously treated cases, 75% of them were proven to be microbiologically relapsed in our study. Multi drug resistant patterns were shown in 31.2% of the total relapse cases by phenotypic DST

method. A mono resistance pattern to INH was shown by Genotype MTBDRPlus assay in one participant who discontinued the treatment regimen. A sensitive DST result were shown in 43.7% of the participants with relapse. This might be due to non-compliance of the anti-TB treatment regimen by participants with relapse claiming they finished their full course of treatment.

In our study, the overall rate of MDR among the entire participants were 3.2%. A study from Ethiopia conducted on stored isolates has shown a lower rate of MDR, 2.2%.[28] The inconsistency might be due to the fact that the later study was conducted on the stored isolate collected in an older period when compared with the current study. This might indicate an alarming increase of drug resistance in TBLN patients. In this study, Genotype MTBDRPlus has detected four MDR cases and one mono resistant to INH. The later mono resistance to INH was inconsistent with the finding from BACTEC MGIT 960 employed in this study, showing five MDR cases. But Cohen's kappa has shown a perfect agreement between the two methods. A similar trend of perfect agreement between the two methods was shown by one study conducted in Ethiopia.[29]

The other molecular diagnostic tool employed in this study was GeneXpert MTB/RIF assay, which showed a detection rate of positive cases in 33.3% of the total participants enrolled in this study. A slightly similar report of 39% has been shown by one study conducted in Ethiopia.[30] The rate of RIF resistance by GeneXpert MTB RIF assay in our study was 9.5% (4/42) among GeneXpert MTB RIF positive cases or 3.2% (4/126) among the entire participants. This finding, among the GeneXpert cases, is much higher than the finding from a study conducted in India which showed a RIF resistance rate of 4.7% (2/42). But the overall RIF resistance, or 3% (2/67), is almost similar.[31] A RIF resistance pattern of 7.8% (7/89) among GeneXpert positive cases were shown by another report, which happened to be again lower than the finding from this study. But the overall RIF resistance pattern, 4.8% (7/145), was shown to be slightly higher than the report from this study.[32] When compared with BACTEC MGIT 960, GeneXpert MTB RIF assay missed one RIF resistant case. A slightly similar RIF resistance pattern was shown by one study conducted in United Arab Emirates.[33] GeneXpert MTB/RIF assay was used when employed as a diagnostic tool for TBLN suspected patients, and proved that it is an excellent tool provided that it also generates results regarding the drug resistance pattern, particularly in those TBLN patients with recurrent infection. The finding from this study has indicated that diagnosing RIF resistant cases by GeneXpert MTB/RIF assay and with phenotypic DST for further testing of first line drugs has considerable importance, particularly for those patients who have taken a full course of anti-TB regimen and revisiting the clinic once again with enlarged lymph node and other TB like presentations. As the finding from this study has shown, in a resource constrained country, where there are a limited supply of molecular DST and phenotypic methods, focusing on previously treated cases would help in the efficient utilization of the available supplies. In addition, this study tried to generate evidence about the non-tuberculous causes of lymph node enlargement following the completion of a full course of anti-TB treatment.

This study has a resource limitation and as a result further molecular characterization such as Spoligotyping was not performed.

Conclusion

Overall, a considerable microbiological relapse was observed among previously treated TBLN cases and, most importantly, a substantial number of either mono or multi drug resistance was observed among these patients. Our findings are as expected with a higher proportion of resistance seen among previously treated TBLN patients and we strongly suggest that those with relapse need to be screened for drug resistance either using rapid molecular tests or by phenotypic methods. Furthermore, no bacterial or fungal growth, other than MTB, was observed as a cause of lymphadenitis in the studied population.

Recommendation

We strongly recommend, due attention should be given to diagnose EPTB patients, particularly TBLN cases with prior history of treatment. Early screening of TBLN patients for MDRTB is crucially important for appropriate TBLN therapy.

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Disclosure

The authors report no conflicts of interest in this work.

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