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Isolation of Diverse *Mycobacterium Tuberculosis* Strains Employing Automated and Conventional Culture from Lymphadenitis in a Tertiary Care Center, Pondicherry, India

Kandhakumari Gandhi[1], Stephen Selvaraj[2*], Kavitha Kannaiyan[3]

[1]*Department of Microbiology, Kanti Devi Medical College and Research Centre, Mathura, Uttar Pradesh, India*

[2]*Department of Microbiology, Mahatma Gandhi Medical College & Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University), Pondicherry, India*

[3]*Department of Microbiology, Aarupadai Veedu Medical College and Hospital, Pondicherry, India*

ABSTRACT

In India, tuberculosis remains a major public health problem and Tuberculous lymphadenitis remains a diagnostic challenge. This study aimed to isolate M. tuberculosis from lymphadenitis employing LJ and MGIT culture. A total of 49 single clinical specimens collected from patients with clinical suspicion of Tuberculous lymphadenitis were included. Ziehl-Neelsen microscopy and culture was performed and growth of M. tuberculosis was identified using standard tests. Drug susceptibility testing was carried out in MGIT and the isolated M. tuberculosis strains causing lymphadenitis were genotyped by spoligotyping. Among 49 samples processed, 28.6% were positive by ZN, 20.4% on LJ and 26.7% in MGIT. LJ and MGIT together yield 32.7% positivity. The mean and median turnaround time for LJ and MGIT culture was 31.8 and 14.5 days respectively. Conventional identification tests identified 15 M. tuberculosis strains and one M. bovis BCG. Drug susceptibility revealed 50% isolates were resistant to one or more drugs, but not MDR-TB. Spoligotyping revealed 47% of the strains causing lymphadenitis were profiles described as orphans. To conclude, inclusion of both LJ and MGIT increases percentage positivity. The short Turn Around Time (TAT) of MGIT helps early reporting of drug susceptibility thus avoiding empirical treatment, in areas where molecular techniques are not feasible. Orphan spoligotype is associated with lymphadenitis.

Key words: *M. tuberculosis*, Lymphnode, Lowenstein Jensen, MGIT960, Lymphadenitis

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Corresponding author: Stephen Selvaraj
e-mail ✉ : stephens4950@gmail.com
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INTRODUCTION

In developing countries like India where the incidence of Tuberculosis (TB) is high, TB of the lymph node (*Tuberculous lymphadenitis*) remains the most common form infecting 30%-52% among extrapulmonary tuberculosis cases [1]. Lymph node tuberculosis occurs due to lymphatic dissemination of *Mycobacterium tuberculosis*. The infected lymph nodes in the later stage may break open discharging pus and ultimately the wound will not heal for several years. Lymphadenitis is very common in childhood and commonly reported from females when compared to the male. But today lymphadenitis has been reported from people between the age group of 20 years to 40 years [2]. Various conditions like viral or bacterial adenitis, fungal disease, toxoplasmosis, cat scratch fever, carcinoma and other conditions present the same cytology or histopathology that mimics granulomatous lymphadenopathy. These require the differential diagnosis to distinguish them from *Tuberculous lymphadenitis*. Presence of acid fast bacilli (AFB) in smear and isolation of *M. tuberculosis* from lymphadenitis pus is the standard diagnostic method [3]. Due to limitations with conventional diagnostic techniques and extensive differential diagnosis, conditions like *Tuberculous lymphadenitis* need a diagnostic tool which is highly sensitive and specific. Smear microscopy is less sensitive and in developing countries like India, Lowenstein-Jensen (LJ) culture remains the gold standard, although it is time consuming. However automated liquid culture systems like Mycobacterium Growth Indicator Tube (MGIT 960) have been reported to have better sensitivity with short turnaround time compared to solid culture. Spacer oligonucleotide typing (Spoligotyping) is a polymerase chain reaction (PCR)-based genotyping method which detects and differentiates strains of

Mycobacterium tuberculosis complex based on DNA polymorphism present at the "Direct Repeat" (DR) region. The DR region is made up of one or more IS6110 elements and consists of direct repeat sequences which are interspersed by non-repetitive DNA spacers. Presence or absences of DNA spacers in the DR region helps to characterize the diverse strains and to understand the epidemiology. So far, studies on *M. tuberculosis* strains causing *Tuberculous lymphadenitis* are not available. Hence in this study, we aimed to isolate *M. tuberculosis* from lymphadenitis sample employing LJ and MGIT and to study the genotypic diversity by spoliotyping.

MATERIALS AND METHODS

This work has been carried out in a tertiary care super specialty teaching hospital at Pondicherry. Written informed consent was obtained from patients and the work was approved by Institutional Human Ethical Committee. Patients with pulmonary tuberculosis, sputum positive cases and those treated with Anti-tuberculous treatment drugs were excluded from the study. Forty nine single specimens collected from patients who presented with enlarged superficial lymph nodes for more than 1 month duration with or without other constitutional symptoms like fever and weight loss were included in this study. All 49 samples were processed for smear microscopy and LJ culture. However, only 45 samples were processed in MGIT due to insufficient quantity. With sterile precautions, specimens were processed for direct Ziehl-Neelsen (ZN) staining without decontamination. For culture, the specimens were digested and decontaminated by NaOH-NALC (Sodium hydroxide-N-acetyl-L-cysteine) procedure before inoculation. The inoculated LJ and MGIT tubes were monitored for eight and six weeks respectively. Growth of *M. tuberculosis* complex (MTBC) recovered in LJ and MGIT was identified and confirmed following standard protocols [4]. Drug susceptibility testing was carried out for five first line drugs (Streptomycin, Isoniazid, Rifampin, Ethambutol and Pyrazinamide) in MGIT following the recommended procedure of the manufacturer. Reference strain of *M. tuberculosis H37Rv* was used as control. The different strains of *M. tuberculosis* isolated from lymphadenitis cases were spoligotyped at National Institute of Research in Tuberculosis, Chennai following the procedure of Kamerbeek et al. [5].

RESULTS

Among the 49 samples included 16 samples were positive by culture. Out of these 16 patients 13 (81.3%) were male and 3 (18.7%) were female. The median age of the patient was 26 years (range 1 year to 68 years). ZN microscopy was positive for AFB in 28.6% (14/49) cases, LJ positivity among 20.4% (10/49), and MGIT positivity in 26.7% (12/45) cases. Presence of AFB growth on LJ and/or MGIT was considered as culture positive. Culture grew *M. tuberculosis* complex (15 strains of *M. tuberculosis* and one strain of *M. bovis* BCG) in 32.7% (16/49) of the samples processed. All the isolates which have grown on LJ were positive by smear. But four isolates which were positive by smear have not grown in LJ, but isolated using MGIT. In our study two smear and LJ culture negative specimens have grown exclusively in MGIT. In this study six isolates which have not grown in LJ has been isolated using MGIT. But on the contrary two smear and LJ positive isolates have not grown in MGIT.

The mean turnaround time for LJ culture positivity from lymphadenitis pus was 31.8 days and the median for BACTEC MGIT 960 TB culture was 14.5 days. Growth on LJ and in MGIT tubes by smear positive and negative cases are presented in Table 1. All sixteen isolates were identified as MTBC by using SD Bioline immuno-chromatographic rapid kit for production of MPT64Ag. All isolates were sensitive to Para nitro benzoic acid (PNB) susceptibility assay in MGIT960. Conventional tests like Acid fastness, Cord factor, Niacin production and Nitrate reduction carried out identified 15 isolates as *Mycobacterium tuberculosis* and one isolate as *Mycobacterium bovis*. Spoligotyping characterized *M. bovis* as a BCG vaccine strain. Spoligotyping technique, besides serving as an epidemiological tool for genotyping study, also serves as an identification tool in the differentiation of species within the *M. tuberculosis* Complex, which further confirmed the identification by conventional biochemical tests.

Among 16 isolates subjected to anti-mycobacterial drug susceptibility testing 8 (50%) were sensitive to all first line drugs tested. Two isolates showed dual resistance to streptomycin and Isoniazid, whereas two isolates showed mono-resistance to Isoniazid. Isoniazid resistance with or without combination with other drugs was seen in four isolates. One strain of *M. bovis* BCG showed natural resistance to pyrazinamide. Mono-resistance to Rifampicin, Ethambutol and Pyrazinamide each was seen in one isolate respectively. The different spoligotypes from lymphadenitis pus is given in Table 1 [6].

Table 1: Different laboratory parameters of *M. tuberculosis* and *M. bovis* BCG strains isolated from lymphadenitis

S No.	Age (year)/Sex	ZN	LJ	TAT-LJ (Days)	MGIT	TAT-MGIT (Days)	AFB in culture	Niacin	Nitrate Reduction	MPT64Ag	Streptomycin	Isoniazid	Rifampicin	Ethambutol	Pyrazinamide	Spoligotype

1	37/M	+	+	21	ND	-	+	+	+	+	S	S	S	S	S	EAI6_BG D1
2	25/M	+	+	18	ND	-	+	+	+	+	S	S	S	S	S	U
3	38/M	+	NG	NG	+	20	+	+	+	+	R	R	S	S	S	BEIJING
4	12/M	-	NG	NG	+	14	+	+	+	+	S	S	S	S	S	Orphan
5	18/F	+	+	32	+	15	+	+	+	+	S	S	S	S	S	EAI6_BG D1
6	4/M	+	+	32	+	15	+	+	+	+	S	S	S	S	S	Orphan
7	27/M	+	NG	NG	+	55	+	+	+	+	S	R	S	S	S	Orphan
8	28/M	+	+	56	+	12	+	+	+	+	S	S	S	S	S	Orphan
9	12/M	+	NG	NG	+	12	+	-	-	+	S	S	S	S	R	BOVIS1_B CG
10	19/M	+	NG	NG	+	12	+	+	+	+	R	R	S	S	S	U
11	1/M	+	+	40	+	56	+	+	+	+	S	S	S	S	S	Orphan
12	52/F	+	+	28	-	0	+	+	+	+	S	S	S	S	R	BEIJING
13	27/M	-	NG	NG	+	13	+	+	+	+	S	R	S	S	S	Orphan
14	35/M	+	+	28	+	16	+	+	+	+	S	S	S	S	S	U
15	50/M	+	+	35	+	12	+	+	+	+	S	S	S	R	S	Orphan
16	38/F	+	+	28	-	0	+	+	+	+	S	S	R	S	S	ND

TAT-Turnaround Time, NG-No Growth, ND-Not Done, S-Streptomycin, I-Isoniazid, R-Rifampicin, E-Ethambutol, PZA-Pyrazinamide

DISCUSSION

Tuberculous lymphadenitis is caused mainly by *M. tuberculosis* and non tuberculous mycobacteria in developing countries like India and other developed countries. In our study we have isolated 15 strains of *M. tuberculosis* and one *M. bovis* BCG vaccine strain, which was isolated from a case of lymphadenitis. This child was vaccinated with BCG. BCG vaccines are given as a protective measure against severe forms of EPTB such as TB meningitis and hence infants are vaccinated with single dose of Bacille Calmette-Guérin (BCG) vaccine after birth according to childhood immunization programmes in high TB burden countries. Conventional identification techniques are capable of recovering the organism as only *M. bovis*. But spoligotyping identified it as a vaccine strain of *M. bovis* (BOVIS1_BCG). Hence spoligotyping differentiated the animal strain *M. bovis* and the vaccine strain, and hence epidemiological tools like spoligotyping are useful for the proper characterization of strains.

In India, Ziehl-Neelsen microscopy is widely used for diagnostic purpose. The sensitivity of this microscopy varies depending upon the source of sample. Unlike sputum specimen, extrapulmonary specimens like pus, and body fluids like Cerebrospinal fluid, pleural fluid, synovial fluid etc., are paucibacillary. Ghariani et al. [7] reported only scanty bacilli from 75.6% of the smear positive extrapulmonary specimens. Pus collected from lymph nodes yield increased positivity by smear microscopy compared to body fluids. Handa et al. [2] reported increase in AFB positivity smears prepared from purulent aspirates. AFB smear positivity in lymphadenitis sample ranges from 15% to 47% depending on the presence of necrosis [1,8]. Mittal et al. [9] reported the sensitivity and specificity of AFB smear microscopy from lymph node aspirates were 76.47% and 100% respectively. Studies have reported that concentration techniques increase the sensitivity. Patwardhan et al. [10] reported 30.7% from lymphadenitis samples while using direct microscopy and 41.5% using concentrated ZN technique. Mirza et al. [11] reported 42% smear positivity from cytology positive lymphadenitis patients. Reddy et al. [12] reported 12% smear positivity from tuberculous lymphadenitis and 0% from granulomatous lymphadenitis. Another study by Sharma et al. [13] reported 30% and 0% positivity from confirmed and suspected case of lymphadenitis respectively. The present research recorded 28.6% using smear microscopy.

Patwardhan et al. [10] reported LJ positivity from lymphadenitis samples ranging from 14% to 57%. Presence of necrosis increases the culture positivity ranging from 35% to 65% [1]. The highest isolation rate of 50.7%, followed by 45% and 32.6% positivity using LJ was reported by few authors [10,12,14]. In the present study, 20.4% LJ positivity was reported among the 28.6% AFB smear positive cases. All the isolates which have grown on LJ were smear positive and nearly 28.6% (4/14) of the smear positive cases failed to grow on LJ. Among the total culture positive samples only 62.5% isolates have grown in LJ and others were isolated using MGIT system.

Gautam et al. [15] reported 18% positivity using MGIT from lymphadenitis specimens. Four smear positive isolates which have not grown in LJ was isolated using only MGIT system. Also two smear and LJ culture negative specimens have grown exclusively in MGIT. Thus, six isolates which have not grown in LJ were isolated using MGIT. On the contrary, two smear positive and LJ positive isolates have not grown in MGIT and have grown only in LJ which would have been missed if LJ is not included.

The mean TAT (Turn around Time) on LJ was 26 days and 30 days and in MGIT it was 13 days and 20 days in case of smear positive and negative specimens respectively [10]. In our study the mean TAT for LJ and was reported as 31.8 days and the median TAT for MGIT was 14.5 days respectively. So MGIT is far superior to LJ in terms of higher yield and short turnaround time. Hence from purulent pus materials, the positivity of AFB in ZN smear and liquid culture is quite high. Thus automated liquid culture system can be very helpful for optimal isolation of Mycobacterium from pus sample unlike body fluids.

Drug resistance is very common in *M. tuberculosis* isolated from pulmonary specimens than extrapulmonary. DST for *M. tuberculosis* isolates from TB lymphadenitis show different levels of resistance. Thus Zewdie et al. [16], from Ethiopia observed that 8.3% of their isolates from TB lymphadenitis were MDR TB and 1.7% showed mono-resistance to any one of the four first line drugs. According to an earlier report by Biadglegne et al. [17], 8.1% of resistance was recorded comprising 1.4% MDR-TB and 6.7% showing mono-resistance. Recently Sharma et al. [18], reported that among their 63 isolates of MTB from TB lymphadenitis, six were MDR TB and two were INH mono-resistant, with a percentage resistance of 8.7%. A supranational reference laboratory from India by Dusthackeer et al. [6], has reported a very high resistance of 57.0% based on a large number of isolates from Lymph nodes. They recorded MDR TB of 19.0% and 38.0% isolates showing mono-resistance. This report which is made from a Tuberculosis Research Laboratory at Chennai, India, which is about 160 Km away from Pondicherry, is very much similar to our present study.

Salvador et al. [19] has discussed about the *M. tuberculosis* strains and its association with specific sites. Sankar et al. [20] from North India reported the association of Manu strains with extrapulmonary tuberculosis. In our study majority, around 47% of *M. tuberculosis* strains were profiles described as orphans, which may indicate their association with lymphadenitis cases. No specific spoligotypes were seen in specific age or sex groups.

CONCLUSION

In TB endemic countries, TB lymphadenitis remains the most common extrapulmonary manifestation of tuberculosis. High index of clinical suspicion and diagnostic tests with high sensitivity and specificity helps in the definitive diagnosis of lymphadenitis. Combination of conventional and automated tests increases the percentage positivity. Automated systems like MGIT960 can differentiate the live and dead bacilli, helps to detect drug resistance and serves as a right tool for proper management of patient by avoiding empirical treatment. In our study majority, around 47% of *M. tuberculosis* strains were profiles described as orphans, which may indicate their association with lymphadenitis cases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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