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Original Article

Diagnosis of Genial Tuberculosis by PCR and other conventional methods

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Abstract

Female Genital Tuberculosis is a unique diagnostic challenge among the infertile population in countries where TB poses a high epidemiological risk. Early diagnosis and effective management is crucial to secure the reproductive organs from irretrievable damage. This cross sectional study was done between April 2011 to March 2012 in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University in collaboration with National Tuberculosis Reference Laboratory and TB laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh to compare the different laboratory tests on endometrial tissue for the diagnosis of genital tuberculosis. Laparoscopic endometrial tissue biopsies of 91 infertile women with clinical suspicion of Genital Tuberculosis were subjected to AFB smear microscopy, culture in Lowenstein Jensen media, histopathology and PCR assay. Two primers - *mpt64, IS6110* were used as target for *Mycobacterial* DNA. Among the endometrial tissue samples of 91 infertile cases, AFB smear was positive in 43 (47%) cases, 8 (7.6%) cases was positive in culture; while PCR was positive in 36 (42.4%) cases. None of the cases were positive for epithelioid granulomatous lesions. PCR was found positive in 33% of smear negative cases and 35% of culture negative cases while PCR was found negative in 53.4% of smear positive cases and 12.5% of culture positive cases. Use of both AFB smear microscopy and PCR assay enhances the detection rate of GTB.

Key words: Female genital tuberculosis, infertility, PCR

Introduction:

Tuberculosis is still rampant in Bangladesh and ranks 6th on the list of 22 highest TB burden countries in the world [1]. Although pulmonary TB (PTB) remains the commonest and the most infectious type of TB, extra-pulmonary TB (EPTB) is becoming more prevalent especially in young women throughout the world [2]. Genital TB (GTB) is an uncommon form of extra pulmonary TB (EPTB) that often remains undiscovered [3]. Females with genitourinary TB comprise approximately 0.5% of all TB cases, out of which near about 50% are GTB cases[4]. Being usually torpid, it takes years to manifest clinically after initial seeding[5,6]. Female genital tuberculosis (FGTB) is one of the important causes of infertility and most cases are secondary to pulmonary or abdominal tuberculosis[7,8,9]. The fallopian tube is the initial site of involvement, followed by endometrium in 50-90 % of cases [10,11]. Clinically, FGTB may remain asymptomatic (11%) and 40-50% cases present with infertility[12].

Diagnosis of FGTB is often missed due to its asymptomatic presentations and nonspecific complains thus entails a combination of patient's clinical portrait and laboratory findings. Direct demonstration of the TB bacilli under microscope by AFB staining of the endometrial tissue, is currently the most rapid and definite method of detection. But its sensitivity is low since at least 10^4 bacilli/ ml must be present in the specimen to give a positive result[13]. Though culture of TB bacilli is more sensitive, requiring as little as 100 organisms/ ml and gold standard for diagnosis of TB elsewhere, but it is not so incase of FGTB. Several study shows that only 3-8% of clinically suspected cases were culture positive among the infertile women[9,14]. Histopathological examination of the endometrial tissue for the presence of TB granuloma is universally accepted as an

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adequate tool for FGTB diagnosis but may also present with other features[15]. In recent years, polymerase chain reaction (PCR) technique has evolved as a useful and rapid technique for the diagnosis of EPTB[16]. PCR can detect quiescent or early disease before symptoms are evident[16,17]. Several studies detected 13-56% PCR positive cases among infertile women9,[14,18,19]. FGTB has a tremendous impact on reproductive health. Early diagnosis is indispensable to ensure early treatment and restore fertility. Therefore, the study was designed to compare the different diagnostic methods for GTB.

Methods:

This was a cross sectional study conducted in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. A total of 91 infertile women with clinical suspicion of GTB attending Infertility Wing (BSMMU) and Care Hospital during the period of April 2011 to March 2012 were enrolled for laparoscopic endometrial biopsy. Women with established causes of Infertility like endometriosis, Polycystic Ovarian Syndrome (PCOS), Hyperprolactinemia, structural problems causing obstruction like- inborn abnormalities (mullerian agenesis) were excluded from the study. Laboratory works were performed in the Department of Pathology, BSMMU, National Tuberculosis Reference Laboratory (NTRL) and at the TB laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B).

Clearance from the Institutional Review Board was obtained to conduct the study and informed consent was obtained from each patient. Purposive sampling was done. A predesigned data sheet along with past medical records of infertility investigation of the study population were collected

Sample processing:

Samples were collected in normal saline, processed and Ziehl-Neelsen's staining was done. Remaining part of the sample was decontaminated using 4% NaOH followed by centrifugation. The concentrated sediment was used for culture in Lowenstein-Jensen media at 37^{o}C and PCR assay. Growth was monitored for 8 weeks and confirmed by Z-N staining and biochemical tests. Histopathological slides were stained with hematoxylin-eosin and observed for the presence of granuloma with or without caseation and Langerhan's giant cells.[15,20,21]

DNA extraction:

DNA was extracted from the templates by heat and sonication method at 95^{o}C in a water bath for 20 minutes followed by ultrasonication bath for 15 minutes.

PCR assay:

Identification of M.tuberculosis was done by using 2 pairs of primers IS6110 and mpt64.

An IS6110 fragment of 123bp length was amplified using primers IS6110 a (5'-CCTGCGAGCGTAGGCGTCGC-3') and IS6110b (5'CTCGTCCAGCGCCGCTTCGG-3'0) while mpt64 primers: MPT1 (59-TCCGCTGCCAGTCGTCTTCC-39) and MPT2 (59-GTCCTCGCGAGTCTAGGCCA-39) amplified a fragment of 240 bp length. The PCR was carried out in 25 µl volume consisting of 5µl template DNA and 20 µl PCR mixture (Master Mix) consisting of 5X PCR Buffer,50mM MgCl2,10mM dNTPs,0.4mM of each primer,1 .25 Units of Taq DNA polymerase. For a positive control, DNA of H37RV strain was used and PCR grade water, as the negative control. Using a thermal cycler (MJ Research, PTC-200 Peltier Thermal Cycler) amplification was carried out for IS6110 consisting of denaturation at 94^{o}C for 45 sec, annealing at 68^{o}C for 45 sec, extension at 72^{o}C for 2 min), and for mpt 64 consisting of denaturation at 94^{o}C for 30 sec, annealing at 57^{o}C for 45 sec, extension at 72^{o}C for 30 min. Detection of amplified products was done by agarose gel electrophoresis (on 1.5% Agarose gel (Ultra pure Tm Agarose, Invitrogen) at 60 volts for 1.5-2 hours. Gel was stained with ethidium bromide and viewed under UV transilluminator using a gel documentation system (BIORAD, Italy).

Results:

Most of participants were found in the age group 26-30 (38.5%) and resided in urban areas (66%). Maximum participants 37 (40.7%), had no complains other than infertility. Duration of infertility was between 3-4 yrs among the majority 29 (33%) of the study population. Symptomatic patients suffered mostly from dysmenorrhoea 26 (28.9%) followed by irregular menstruation 17 (18.7%) (Table -1).

Among the 91 patients, 5 (5.5%) patients had history of TB in the past and Mantoux test was positive in 4 (4.4%) cases. A raised ESR level was present in 8 (8.8%) cases.

Out of a total of 91 suspected cases of GTB, 43 (47%) cases were positive by AFB smear microscopy but only 8 (7.6%) cases were positive by culture. PCR was positive in 36 (42.4%) cases. None of the cases were positive for granulomatous lesions. Chronic inflammatory cells were present in 8 cases among which, PCR was positive in 3 cases (Table -II).

Among the 39 cases that were smear positive but culture negative, 17 (47.2%) cases were also PCR positive. There were 12 (33.3%) PCR positive cases, which were both smear

and culture negative. However, 32 (58.1%) cases were negative by all the three tests(Table -III)

On comparison of the bacteriological test results with PCR, it is seen that out of a total of 43 smear positive cases, PCR was positive in 20 (46.5%) cases with a significant association. Among the 8 culture positive cases, PCR was positive in 7 (87.5%) cases with a high significant association (Table IV). All these cases were positive using the primer mpt64 only. Out of the 36 PCR positive patients, 18 (50%) patients were detected using the primer mpt64 and 16 (44.4%) patients using the primer IS6110. Only 2 (5.5%) cases were detected by both the primers.

Table -IDistribution of the study population according to symptoms other than infertility (N= 91)

Symptoms	Number of cases	Percentage
Dysmenorrhoea	26	28.6
Irregular menstruation	17	18.7
Amenorrhoea	06	7.0
Oligomenorrhoea	05	5.5
Asymptomatic	37	40.7
Lower abdominal pain	04	4.4
Lower abdominal mass	01	1.1
Fever	01	1.1

* Multiple responses

Table- II Result of laboratory methods for MTB among study cases (N=91)

Tests	Number of positive cases	Percentage
AFB smear microscopy	43	47
Culture of MTB	08	7.6
Histopathology	00*	00
PCR	36	42.4

* Epitheloid granuloma or tubercles were not seen in any case. Chronic inflammatory cells were seen in 8 cases. Rest had normal findings.

Table- III Comparison of AFB microscopy, culture and PCR assay among study cases (N=91)

Bacteriological tests	Total	PCR result	
		Positive	Negative
Both AFB microscopy and culture positive	04	03	01
AFB microscopy positive and culture negative	39	17	22
AFB microscopy negative and culture positive	04	04	00
Both AFB microscopy and culture negative	44	12	32
Total	91	36	55

Table- IV Correlation between AFB microscopy, culture and PCR assay among study population (N=91)

PCR	AFB		Culture	
	Positive(n=43)	Negative(n=48)	Positive(n=08)	Negative(n=83)
Positive	20 (46.5%)	16 (33.3%)	7 (87.5%)	29 (34.9%)
Negative	23 (53.4%)	32 (66.7%)	1 (12.5%)	54 (65.1%)

Discussion:

Female Genital Tuberculosis is a unique diagnostic challenge among the infertile population in countries where TB poses a high epidemiological risk. Early diagnosis and effective management is crucial to secure the reproductive organs from irretrievable damage. The paucibacillary nature and asymptomatic presentations make the diagnosis intricate. Molecular techniques targeting mycobacterial DNA or RNA in the recent years have proved to be very expedient in diagnosing GTB.

The present study includes 91 infertile women clinically suspected of GTB undergoing laparoscopy. The samples were subjected to AFB smear microscopy, culture for MTB in L-J media, histopathology and PCR. Two sets of primers were used to arbitrate a better target for PCR amplification- *Mycobacterium tuberculosis* specific primer- *mpt64* and *M.tuberculosis* complex specific primer- IS6110.

FGTB occurs in young women in their reproductive years. In the present study, maximum number of patients was in the age-group 26 to 30 which is comparable to an Indian study who found the range between 20-35 years[16].

The duration of infertility ranged from minimum of 1 year to the maximum of 13 years. This is comparative to the study by Vidushi and his group where the duration ranged from 12 months to 20 years[22].

Majority of the patients were in the asymptomatic group 37 (40.7%) which is similarly noted[12]. Among the other symptoms, dysmenorrhoea was the most (28.6%) frequently stated complain. FGTB is secondary to disease elsewhere, frequently the lungs, in most cases. In this study, 3 out of 5 women with past history of tuberculosis had positive PCR results. This result is comparable to the study done in India[16].

Out of 91 study samples, 47% of the endometrial biopsies were found AFB smear microscopy positive. This was similar to a study done in Bangladesh [23]. On the contrary, an Indian study showed 8.3 % AFB positive cases among 65 infertile women[16]. Staining by Acid fast technique cannot differentiate live from dead bacilli. Moreover, atypical mycobacteria also take the Z-N stain. Besides *M.tuberculosis*, other species like

M.bovis, M.abscessus, M.chelonae, have also been isolated from the female genital tract in several studies [8,24].

Culture positive for *M.tuberculosis* from the endometrial tissues of the study population accounts for 7.6 %. The reasons for this low positivity could be due to paucibacillary load in samples like endometrial tissue. Furthermore TB bacilli might be completely absent in the part of the sample selected for culture. In an Indian study, it was reported that a substantial number of TB lesions of the genital tract is bacteriologically mute[9]. Moreover, decontamination procedure also reduces culture sensitivity to an extent [25]. Poor rates of isolation (3.3-10.6%) by culture have been reported elsewhere [16,26].

Histopathological evidence for the presence of granuloma was not seen in the present study which is similar to an Indian study[9]. The possible reasons could be that the sampled site may not represent the infected area. The fallopian tubes are the initial site of involvement of the TB bacilli followed by endometrium and so the infected site can be easily missed. Moreover, the time for granuloma formation is often inadequate due to the cyclical shedding of the endometrium[16]. In a 12 year study it was observed that tuberculous endometritis may have any endometrial patterns-proliferative, secretory mixed or hyperplastic which is compatible with the present study[21].

Among the 91 samples, PCR showed a positivity rate of 42.4%, using the primers, *mpt64* and IS6110. This is comparable with the study done in India showing 36.7% PCR positivity[16]. Several studies performed on endometrial specimen have documented the range for PCR positivity varying from 22.5 % to 64.7 % [8,18,27,28,29].

The oligonucleotide primers derived from *mpt64* and IS6110 were used to amplify a 240 bp a 123bp DNA sequence belonging to MTB and *M.tuberculosis* complex respectively. Majority of the PCR positive cases were detected by *mpt64*. Concomitant results were shown by two other studies [9,17].

Positivity rate of IS6110 was low as compared to *mpt64* in this study. Till date, there are no such reports from Bangladesh regarding this strain that may be a reason for the low positivity of IS6110. Besides, studies comparing *mpt64* and IS6110 in the diagnosis of GTB have not yet been commenced. All the culture positive cases were detected by *mpt64*. There were only two cases that could be detected by both the primers showing an increase in positivity by combination of primers.

In this study, PCR test result had significant association with the results of AFB staining and culture. Further, it was also noticed that one third of the AFB smear and culture negative cases were PCR positive which implies that without PCR these cases would have been missed. So, for diagnosing GTB, conventional tests along with PCR, reduces the chance of missing a positive case.

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