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Research Article
Study on Conventional and Molecular Diagnostic Tools for Genital Tuberculosis Associating with Infertility in Indian Women

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Abstract

Background and Objective: *Mycobacterium tuberculosis*, the etiological agent for tuberculosis has been comprehensively studied for over a century now. But the disease still remains a major public health concern today in the 21st century. The précised and authenticated diagnosis is mandatorily pre-requisite to proper medication and cure of tuberculosis that specifically interferes and thus complicating physiological and biochemical mechanism of setting pregnancy in Indian women ranging between the ages of 18-40 years. **Materials and Methods:** Out of 700, 400 females were finally recruited on the basis of inclusion and exclusion criteria. Further, various combinations of conventional and modern diagnostic tools were considered and monitored for the best possible option supported by statistical analyses. **Results:** It was observed that all the 37 laparoscopy positives were also deoxyribonucleic acid-polymerase chain reaction positive during the present study. **Conclusion:** Data obtained from the present study provides new insights into polymerase chain reaction with modified and advanced protocol likely to be competent as a novel molecular diagnostic technique for rapid and précised diagnosis of genital tuberculosis causing infertility in developing countries.

Key words: Amenorrhea, Endoscopy-laparoscopy, genital tuberculosis, hysteroscopy, menorrhagia, *Mycobacterium tuberculosis*, oligomenorrhea, polymerase chain reaction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycobacterium tuberculosis, the etiological agent for tuberculosis, has been extensively studied for over a century now. But the disease still remains a major public health concern today in the 21st century. Despite the availability of anti-tubercular chemotherapy tuberculosis still remains a major health problem and is the leading cause of morbidity and mortality in many developing/resource-poor countries. Despite the efforts that are being made to control tuberculosis worldwide, countless numbers of people die with every passing year[1]. Control of the disease is complicated by the fact that one-third of the world's population is latently infected with tuberculosis. Approximately ranging between 5-10% of the dormant infected population has been noticed to proliferate with disease in the course of its life span whereas, the remaining ones function as pathogen-pool consequently reflecting the disease management a noteworthy confront[2].

In addition, the difficulty in co-administration of the anti-TB and anti-HIV drugs as a result of drug-drug interactions is well established. Further, tuberculosis has been observed hard to make a diagnosis in HIV-positive issues through smear microscopy, a pathological investigation extensively practiced in most of the developing countries, ultimately flops to identify in about 80% HIV-positive concerns. It is noticeable that lack of success to improve protocols to diagnose and cure such patients may probably be rather a more costly affair in future, foremost to greater prevalence, larger residence and increased mortality. Rapid, precise diagnosis and speedy healing treatment, under appropriate supervision to confirm that drugs are taken for the suitable duration, is the important to disease control. Amongst several clinical demonstrations vis-α-vis tuberculosis, female genital tuberculosis has been shown to have worldwide apprehension due to a number of connected problems viz., amenorrhea, oligomenorrhea, primary and/or secondary phase infertility, enduring pelvic mass consequent pelvic pain and substantial moratality[3-10]. The disease is being increasingly recognised as a notable cause of infertility in recent years. The factual occurrence of the disease remnants unidentified as the disease has concerns with diagnostic complications largely for the cause that the primary symptoms are generally non-specific and thus infertility is a recognized consequence.

As far as histo-pathological investigation using hematoxylin and eosin especially for granulomatous tissue reactions companionable with tuberculosis is generally indecisive[11]. During the span of last two decades, polymerase chain reaction (PCR) has been recognized as a rapid, precise and sensitive concomitant with molecular specific tool for identification of mycobacterial DNA employing proper amplification protocol equally applicable to pulmonary and extra-pulmonary subjects[12-18]. Besides, latent tuberculosis infection may probably reveal a positive result in PCR based analysis; however, it is noticed to be not worth mentioning because rapid and precise diagnosis is mandatory for prevention of enduring injury to genital organs and resultant state of infertility. To overcome this problem, endoscopic measures viz. Hysteroscopy and laparoscopy are broadly brought into practice in view of investigating women under the state of infertility[19-21]. The PCRs for detection and identification of mycobacteria in clinical specimens have been developed and evaluated[19, 20], but mRNA-based assay offers great promise in differentiating between live and dead bacilli as the average half-life of bacterial mRNA is 3 min[22]. Thus as mRNA is more easily destroyed than DNA, it can distinguish viable from non-viable organism.

Based on the aforesaid facts[23-25], the present study was undertaken to assess the utility of PCR in definitive diagnosis of tuberculosis in Indian infertility patients in conjunction with endoscopic procedures-laparoscopy and/hysteroscopy and conventional tests (smear and culture based on the radiometric BACTEC system). To qualify as a diagnostic test for genital tuberculosis, a protocol of early diagnosis concomitant with high sensitivity and specificity is requisite and authors, thus, carried-out the present study to assess the suitability of PCR as a diagnostic test for female genital tuberculosis and also inter-related the observation of PCR with laparoscopy and other conventional diagnostic tests. Also, considering the clinical importance of multi-drug resistance in genital tuberculosis, authors planned to investigate all genital tuberculosis positive cases for Rifampicin and Isoniazid resistance by automated DNA sequencing of *rpo B*, *kat G* and *inh A* genes.

MATERIALS AND METHODS

Study subjects: A total of 700 females of child-bearing age attending the infertility Outpatient Department were screened at the IVF and Reproductive Biology Centre, Department of Gynaecology, MM Diagnostics and Sri Sai Hospital, Moradabad, India between 2012 and 2015. The ethical committee of MM Diagnostics, Moradabad approved the study. Informed consent was obtained from the patients included in this study.

Clinical cases: Out of 700, 400 females were finally recruited on the basis of inclusion and exclusion criteria.Infertile females presenting with the following findings were included for the

study (1) Women between the age group 18 and 45 years, (2) Women with primary and secondary infertility and (3) Women presenting with unexplained infertility. Whereas, exclusion criteria were as (1) Women with previous history of genital tuberculosis, (2) Women with partner (male factor) infertility and (3) Women who refused to give consent for the study. Samples from 100 healthy fertile females were included to serve as controls. These samples were collected from females attending the family planning OPD for interval ligation and from females visiting the gynaecology OPD with complaints of menstrual irregularities etc. Further, Twenty-five known AFB cultures from different clinical forms of tuberculosis from Tuberculosis Laboratory MM Diagnostics, Moradabad were taken for comparison with molecular tests to ensure the performance of the tests. Diagnosis was achieved through a comprehensive evaluation on the basis of detailed clinical history, physical examination, biochemical investigations, molecular and laparoscopic evaluation. Provisional diagnosis was made through high index of suspicion based on the detailed clinical history.

Laproscopy: All patients underwent diagnostic endoscopy-laparoscopy and/or hysteroscopy under general anesthesia post menstrually using the three puncture technique. During the procedure, features such as tubal blockage/patency, presence of tubercles, peri-tubal and/or peri-ovarian adhesions, granulomas, tubo-ovarian mass, beaded tubes, cornual blockage, caseation, hydrosalpinx, sacculated tubes, signs of chronic in ammation, pelvic in ammatory disease etc were precisely considered.

Conventional coordinated with molecular diagnostic tests: Menstrual blood (MB) was taken in the secretory phase of the menstrual cycle for the second day. Menstrual blood sampling was taken either during menstrual cycle second day in a sterile container with normal saline as a separate procedure. For laboratory processing, the sample will be immediately transported to the tuberculosis and molecular diagnostic laboratory at the MM diagnostics Laboratory Moradabad for further testing and stored at room temperature for conventional tests and at-80°C for molecular tests.

AFB smear: Menstrual blood sample was obtained in normal saline in sterile container. Sample was stored at room temperature till further analysis. The MB sample was ground with sterile mortar and pestle in small quantity of PBS/normal saline. One smear of each specimen was made by spreading the specimen over 2-3 cm in size and is neither too thick nor too thin .The optimum thickness of the smear could be assessed by placing the smear on a printing matter enabling the print readable through the smear. Smear Preparation was done near a flame. This was required, as six inches around the flame considered as a sterile zone which coagulates the aerosol raised during smear preparation. Allowed the slide to air dry for 15-30 min and fixed the slide by passing it over a flame 3-5 times for 3-4 sec each time. The smear was then stained employing the modified protocol of Ziehl-Neelsen (ZN) staining as per protocol described in earlier study[23]. Acid fast bacilli (AFB) were observed as red, beaded rods when examined by ZN stain under oil immersion (100X) lens. The smears were then graded depending on the number of bacilli observed in the stained smear under oil immersion objective lens of light microscope. At least 100 fields were examined before reporting a smear as negative. A repeat smear was made in case of doubtful report and was examined again.

AFB culture: Specimen (0.5 mL) was inoculated in BACTEC (BBL MGIT) medium tube contains modified Middlebrook 7H9 broth base for BACTEC Micro MGIT culture system. The positive cultures were further processed for drug sensitivity by BACTEC MGIT TB system and strain identification was done by p-nitro-α-acetylamino-β-hydroxy-propiophenone (NAP) test using the technique described recently by a research group[24].

Molecular characterization: Polymerase chain reaction process was performed following steps in sequence as (1) Took 25 µL Master Mix (MMX) aliquot in PCR tubes and allowed it to thaw totally if stored in -20°C, (2) Mixed with gentle finger tapping and span shortly to settle down MMX to the bottom of PCR tube, (3) Marked on the tube based on sequence of DNA sample and positive control, (4) Added 25 µL of isolated DNA template and negative control to the MMX, reaching to final volume 50 µL for reaction, (5) Pipetted up and down to mix DNA template with MMX, (6) Span for a second to bring down reaction mixture to the bottom of PCR tube, (7) Kept all tubes into thermal cycler block, already switched on 5 min before starting reaction for auto calibration and (8) Set the program for implication for 40 cycles (Table 1). After completion of amplification keep PCR amplified product in

Table 1: Sequence of temperature in PCR set up

Temperature (°C)	Time (min)	Cycles	Activity
94	6.0	1	Initial denaturation
94	1.0		Denaturation
55	1.5	40	Annealing
72	1.5		Elongation
4			Holding

refrigerator at 4°C until the detection of amplified product. In order to detect the amplified product, a known molecular weight marker (100 bp DNA ladder) was loaded into the first well of 1.6% agarose gel followed by loading of 20 µL of PCR product into each well along with 4 µL of gel loading buffer. Positive and negative controls were precisely loaded with the samples. Electrophoresis was carried out at 100-150 Volt (5-8 V cm^{-1} for 20 cm gel) until the bands in the molecular weight marker were resolved. Later on the gel was examined under UV light (302 nm) on ULTRALUM Electronic UV-Trans-illuminator gel documentation system for the presence of 240 base pair PCR product and photographed.

RESULTS

On the basis of detailed history, the study subjects (400 infertile females fulfilling inclusion criteria) were categorized with regard to their demographic profile, type of infertility and duration of married life. Patients were also categorized according to their gynaecological symptoms, menstrual disturbance, history of contact or past history of tuberculosis and history suggestive of active disease. Findings of molecular tests (DNA PCR, mRNA-based RT-PCR and real-time PCR monitoring in the drug resistant genes) were correlated with a cascade of clinical profile/laparoscopic findings and pregnancy outcome following diagnosis and anti-tubercular treatment.

Demographic profile of study subjects: When the educational and professional/economical background of the patients was compared, it was found that maximum patients (45%) belonged to middle strata of the society while 35 and 20% patients belonged to lower and higher strata of the society as shown in Table 2.

Details of study subjects and healthy fertile controls: The patients in the study group were between the age group 18 and 40 years (Table 3). The mean age of the patients was observed about 28 years with a standard deviation of 4.7 years. On analysis, It is found that maximum number of study subjects (40%) belonged to the age group 26-30 years suggestive of the female genital tuberculosis probably affecting females in their early reproductive age. The duration of marital life ranged between 1.5-17 years. The mean deviation of marital life was 7.2 years with a standard deviation of 3.5 years.

Type of infertility: Primary infertility was noticed in 274 (68.5%) women, while 126 (31.5%) women had secondary infertility (Table 4). Among the patients with secondary infertility, 15.2% had previous living issue, 45.4% had previous abortions and 15.3% had ectopic pregnancy.

Gynaecological symptoms: In the present study, about 52% patients only had infertility and did not have any gynaecological symptoms. Maximum patients (63.5%) had normal menstrual cycles while menstrual disturbances in the form of amenorrhea, oligomenorrhea and menorrhagia were seen in 5, 23.3 and 8.2% patients, respectively (Table 5). Oligomenorrhea was the predominant menstrual disturbance observed in the subjects.

Laparoscopy results among study subjects: Results from diagnostic endoscopy (Laparoscopy) were obtained from 50% of the study subjects (Table 6). A systemic and thorough evaluation of pelvis and abdominal cavity was carried out for evidence of TB and findings such as granulomas, caseation, calcification, tubercles and flocculated as cites were looked for. The fallopian tubes were also evaluated for the presence of

Table 2: Socio-economic status of the study subjects

Socio-economic category	Status of the study subjects (%)*
Lower	20±4
Middle	35±6
High	45±5

*Values are Mean±SD of five replicates

Table 3: Age distribution of study subjects

Age range (year)	Number of cases	Percentage*
18-25	98	24.5±3.5
26-30	107	40.0±5.7
31-35	160	26.8±3.8
36-40	39	8.7±1.4
Total	400	

*Values are Mean±SD of five replicates

Table 4: Type of infertility in patients

Type of infertility	Number of cases	Percentage*
Primary	274	68.5±4.3
Secondary	126	31.5±2.7
Total	400	

*Values are Mean±SD of five replicates

Table 5: Menstrual history of the subjects

Type of menstrual cycle	Number of cases	Percentage*
Normal	254	63.5±4.3
Amenorrhea	20	5.0±0.5
Oligomenorrhea	93	23.3±1.3
Menorrhagia	33	8.2±0.9
Total	400	

*Values are Mean±SD of five replicates

Table 6: Laparoscopy reports among study subjects

Laparoscopic findings	Number of patients
Adhesions	35
Beaded tube appearance	4
Tubo-ovarian mass	8
Tubal block (uni)	48
Tubal block (bi)	32
Tube absent	8
Hydrosalpinx	10
Frozen pelvic	7
Tubercles	4
Fluid in POD	3
Caseous granuloma	5
Endometriosis	10
Calcification	1
PID	5
PCOS	20
Total	200

Table 7: Data of histopathology among the study subjects

Histopathological type	Number of cases	Percentage*
Study group (n = 400)		
Tubercular endometritis	8	2.0±0.2
Chronic endometritis	16	4.0±0.5
Proliferative phase	51	12.8±1.3
Secretory phase	320	80.0±4.0
Hyperplastic endometrium	5	1.2±0.1
Control group (n = 100)		
Tubercular etiology	0	0

*Values are Mean±SD of five replicates

Table 8: Positivity in AFB smear/culture among the study as compared with control groups

Endometrial biopsy	Smear*	Percentage	Culture*	Percentage
Study subjects (n = 400)	8	2	13	3.25
Controls (n = 100)	0	0	0	0

*All AFB smear and culture were also observed to be positive in DNA-PCR

Table 9: Comparison of DNA-PCR results with conventional tests and laparoscopy

Tests	Histopathology	Smear	Culture	Laparoscopy	DNA PCR
Positivity	8/400	8/400	13/400	37/400	51/400

proximal and distal blocks and hydrosalpinx. Presence of adhesions was also noted. Pelvis and peritoneal cavity were also evaluated for presence of other pathology. Presence of fluid in Pouch of Douglas (POD) was also observed. Pelvic pathology like fibroid uterus, endometriosis and polycystic-ovaries was realized as incidental findings.

Histopathological examinations: Pre-menstrual endometrial biopsy samples were taken and subject to histopathology. Out of the 400 women, 2% (8/400) samples showed positive for tuberculosis (Table 7). In these cases, the histology showed sub-epithelial tissue to display multiple caseation granulomas with langerhans giant cells and diffused and dense inflammatory infiltrate comprising lymphocytes, plasma cells and neutrophils. The impression was said to be as granulomatous being highly suggestive of tuberculosis. Another 12 patients showed signs of non-tubercular endometritis.

Smear and culture: Out of the 400 study samples, AFB smear identified 2% (8/400) cases (Table 8). In the control group samples, though none of the samples was observed to be positive. Culture for acid-fast bacillius in BACTEC460TB systems detected 3.25% (13/400) cases. The cultures were identified as positive within 12 days and were identified as MTB on the basis of NAP test. Apart from this, 8 cultures got contaminated. Though, none of the contaminated sample was positive in AFB smear or PCR.

Finally, during correlating the obtained results, it was observed that all the 37 laparoscopy positives were also DNA-PCR positive during the present study (Table 9). Of 37 laparoscopy positives, 4 which initially tested negative or uncertain in DNA-PCR twisted.

Positive in repeat PCR after spiking; later confirmed with DNA sequence homology for 65kDa gene. All these 51 PCR positive samples were confirmed to be true positives by gene sequence analysis of the PCR products as reflected in Table 9.

DISCUSSION

In the present study, authors attempted to analyze the efficiency of various parameters-AFB smear, culture by BACTEC 460 TB systems, HPE, PCR, laparoscopy in the diagnosis of female genital tuberculosis and to develop a test that can be employed for prompt diagnosis of this insidious disease. The study was conducted on 400 infertile subjects is probably the principal study targeting DNA-based diagnostics of genital tuberculosis. During this study, authors also incorporated 100 healthy fertile females as controls, besides 25 AFB cultures from different clinical forms of tuberculosis for comparison to ensure the performance of DNA and mRNA-based molecular tests. All patients also underwent laparoscopy as module of the infertility work-up. The diagnosis of established tubercular disease was lastly based on laparoscopy and PCR since smear and culture revealed poor outcome almost similar to earlier reports[18,21]. Indicating incidence of genital tuberculosis, it has been frequently complicated to determine the accurate level of the problem as the disease originally presents without symptoms and due to diagnostic complications it is singled out only during the study for infertility[26]. Genital tuberculosis is a major causative for tubal infertility in developing countries like India[21,27,28]. The patients considered in the present study were aged between 18 and 40 years of age. The median age of presentation was 28.5±4.9 years. Our study reflects that

genital tuberculosis affects women in relatively young age group as maximum patients belonged to the age group 18-25 years of age showing resemblance to earlier studies[29-31]. This indicates an agreement with the previous studies[12,17,32]. Young patients being affected by genital tuberculosis can be comprehensive by the fact that after adolescence, the blood supply to the genital organs is increased and as a result, more bacilli are capable to achieve the site and cause infection. In such a state, even the dormant bacteria can get reenergized and cause infection leading to additional number of young patients getting afflicted to genital tuberculosis foremost to infertility. Laparoscopy is a significant tool in the assessment of infertility and in the diagnosis of various pelvic conditions. In women with high suspicion of genital tuberculosis, endoscopy aids to obtain microbiological samples under direct visualization, assess the extent of damage and treat the patient accordingly[33-35]. Being a symptomless disease, the symptoms in case of genital TB can vary thus laparoscopy may prove a key tool in its affirmation[20,21].

In-fact, DNA positives in the present study can act as denominator for calculating the efficacy of the laparoscopy that thus was suggestive only in 38 out of 51 DNA-positive cases. Considering laparoscopy as clinical gold standard, the DNA-PCR results of the present study may be well thought-out as the ideal laboratory gold standard, instead of commonly believed culture as the gold standard. Besides, if the figure of 400 patients is taken as denominator of target population, the prevalence of genital tuberculosis in the present study figures-out to be 12.75%. However, this can at best represent 'prevalence within the infertile females' category only and not the prevalence of female genital TB in a particular population of females of child-bearing age. The main advantage of PCR to a rapid, specific and precise molecular technique permitting detection of mycobacteria from both pulmonary and extra-pulmonary specimens within 4-5 h compared to culture with a poor detection rate and requiring a minimum of 12 days to obtain the result[34]. Due to the high sensitivity of PCR, false positivity becomes an issue[35-37]. In the present study, PCR was positive in all the cases with affirmative or suspicious observations on endoscopy while detecting an additional 13 cases which showed normal laparoscopic observations. This could be attributed to the fact while laparoscopy detects conspicuous changes such as pelvic/intra-uterine adhesions, tubercles, beaded tubes, grade III endometriosis with the tubes being the commonest location, subtle changes during the early stage of infection might be overlooked. Thus, an additional 13 women diagnosed with genital tuberculosis by PCR may probably be considered as harbouring sub-clinical or latent infection, the early diagnosis of which could have a significant role in regaining fertility.

Stringent quality control measures were adopted while carrying-out PCR. Several negative controls were interspersed between samples to avoid cross-contamination, thus nullifying false positive results. The sensitivity of PCR has been questionable because of the highly sensitive nature of the technique and certain contradictory reports across the world doubt the PCR positive results. Thus, in view of these contradictions authors were very cautious in ascertaining the efficacy (sensitivity and specificity) before applying in the clinical specimens. Authors addressed this issue by employing various quality control tools. Twenty-five positives in culture controls used in the study also assisted to prove the sensitivity and specificity of the test in the diagnosis of genital tuberculosis. All the samples were subjected to gene sequence analysis and BLAST search, which further confirmed their factual positivity. For ascertaining validity on expectedly negative samples, authors also took samples from 100 healthy fertile females. Four percent (4/100) samples revealed positive results in DNA-PCR. However, none of the samples showed a positive result in mRNA-based RT-PCR indicating that these 4% positives in healthy controls may be the latent infection probably validating tuberculosis being highly prevalent in general population as well in India. Needless to mention that nearly 10% of the world's population is latently infected with tubercle bacilli, hence, 4 otherwise healthy DNA positive females could be harbouring latent/dormant bacteria, rather than assigning these positives as false positives.

Menstrual dysfunction complaints were observed in 36% of patients. Oligomennorhea was found to have a significant correlation between PCR positive and negative women, indicating tuberculosis bacilli to cause scarring of the endometrial lining. Partial or total destruction of endometrium by the disease process resulting amenorrhea has been shown in a few cases. Gross appearance of endometrium was mostly unremarkable. In advanced cases however, ulcerative or atrophic endometrium and an obliterated endometrial cavity due to extensive intra-uterine adhesions was seen on endoscopy. Endometriosis grade II was observed in 21.5% of PCR positive patients. This can be attributed to the fact that endometriosis, being an auto-immune disease, has been shown to create a defect in natural killer activity resulting in decreased cytotoxicity to autologous endometrium. This immune defect may account for the incidence of other infectious diseases including tuberculosis occurring more frequently in these women[37].

CONCLUSION

Conclusively, findings of the present study reveal that PCR-based detection of *M. tuberculosis* in endometrial biopsy specimens is a sensitive technique for pre-emptive vigilance of probable reactivation for genital tuberculosis, a foremost cause of infertility in developing countries. In the absence of a gold standard, PCR may be measured a relatively better diagnostic gold standard for competent diagnosis of genital tuberculosis in view of its high sensitivity as well as specificity. Furthermore, due to performing laparoscopy and other endoscopic procedures on every patient not being feasible in practice, the present study provides new insights into PCR with modified and advanced protocol likely to be competent as a novel molecular diagnostic technique for rapid and précised diagnosis of genital tuberculosis causing infertility in developing countries.

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