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ORIGINAL RESEARCH ARTICLE

Multiplex PCR from Menstrual Blood: A Non-Invasive Cost-Effective Approach to Reduce Diagnostic Dilemma for Genital Tuberculosis

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Abstract

Aim Genital tuberculosis (GTB) is a potent contributor to irreversible damage to the reproductive system and infertility in females. As no gold standard diagnostic tool is yet available, clinical suspicion and relatively insensitive approaches such as histopathology, laparoscopy and hysterosalpingogram are currently critical determinants in the diagnosis of GTB. Although a polymerase chain reaction (PCR)-based assay using endometrial tissue seems promising, sampling does require an invasive procedure.

Objective We hypothesized that menstrual blood may provide an alternate non-invasive source of samples for PCR-based GTB diagnosis.

Methods We enrolled 195 women with primary infertility in whom GTB was suspected. We obtained ethics committee approval from our institution and written informed consent from subjects. Endometrial tissue and menstrual blood was collected from the subjects and culture, histopathology, and multiplex PCR with both sample type was performed for each subject.

Results The sensitivity and specificity of multiplex PCR was, respectively, 90.2 and 86.1% for menstrual blood, 95.8 and 84.3% for endometrial tissue, and 64.8 and 93.2% for histopathology staining.

Conclusions A strong clinical suspicion aided with multiplex PCR using menstrual blood may significantly reduce the diagnostic dilemma for GTB diagnosis in a non-invasive, sensitive, rapid, and cost-effective manner.

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Key Points

Multiplex polymerase chain reaction-based testing using menstrual blood, a non-invasive diagnostic approach to testing for genital tuberculosis (GTB), is likely to be more acceptable to the community than a more invasive approach

Our experimental method demonstrated efficacy over non-specific image-based and other invasive diagnostic procedures

The method seems to be most beneficial for GTB diagnosis in developing countries where varied serological tests, such as the QuantiFERON-TB Gold assay and the Mantoux skin test, are not considered for confirmation of TB diagnosis as they provide significant false-positive results

△ Adis

1 Introduction

Mycobacterium tuberculosis complex (MTBC) is a global burden in the fight against pulmonary tuberculosis (PTB) [1]. Both PTB and extra pulmonary tuberculosis (EPTB) are the leading accelerators for TB-related mortality. In 2014, a total of 1.5 million people died and 9.6 million people were affected by the disease worldwide [1]. The scientific community has already accepted that EPTB is by no means uncommon, particularly in communities where PTB is common [2]. Furthermore, significantly less attention is paid to EPTB than PTB [2, 3]. Among the EPTBs, genital TB (GTB) seems to be less of a focus within the scientific community, but irreversible reproductive damage due to this disease has been documented [2]. The estimated incidence of GTB is < 1% in developed countries but is 18% in India [4]. The prevalence of GTB is probably largely underestimated because of diagnostic difficulties and its complex clinical manifestations. This is despite the fact it was first identified in 1744 when a 20-year-old woman died of GTB and her post mortem revealed evidence of caseous material in her uterus and fallopian tubes [5]. Currently, clinical suspicion aided by intensive investigations are important in the diagnosis of GTB [6, 7]. As an absolute diagnosis from characteristic features in a hysterosalpingogram (HSG) or laparoscopy is not possible, tissue-based culture and histopathological evidence is required [8]. However, these methods are also not sensitive enough to diagnose EPTB with a lower bacterial load [5, 8, 9]. The World Health Organization discourages the use of serodiagnostics, such as interferon-γ release assays (e.g., QuantiFERON-TB Gold), for active TB diagnosis in the developing world because results are inconsistent and imprecise. This represents an additional barrier to the diagnosis of both EPTB and GTB in the developing world, including India [10]. Routine laboratory processes, including a positive chest X-ray for healed or active PTB, contact history, elevated erythrocyte sedimentation rate (ESR), and positive tuberculin test may be indicative; confirmation requires further investigations [8]. In recent years, polymerase chain reaction (PCR) has become a useful and rapid procedure for the diagnosis of both PTB and EPTB, including GTB [11]. The efficiency of the technique is already proven in PTB [11]. PCR-based GTB diagnosis seems promising but requires premenstrual endometrium aspirates or tissue derived from an invasive procedure, either laparoscopy or dilation and curettage (D&C) [8, 9]. Although the PCR-based diagnosis itself is rapid, cost effective and sensitive, significant costs are still involved due to the invasive operative sampling of the endometrium.

The invasive sampling process seems to be a crucial barrier to comprehensive clinic-based screening of suspected individuals, so the diagnostic dilemma persists [5–11]. Given the current diagnostic limitations, we hypothesized that menstrual blood (MB) may be used as an alternative non-invasive sample source for PCR-based GTB diagnosis. To the best of our knowledge, this is the first report to describe the efficacy of multiplex PCR (M-PCR)-based GTB diagnosis using MB instead of endometrial tissue and other conventional methods.

2 Methodology

2.1 Study Subjects

From 2013 to 2016, we enrolled 195 women with primary infertility in whom GTB was suspected based on their symptoms, imaging, and laparoscopic examination. The institutional ethics committee approved the study, and written informed consent was obtained from all patients. We included women who presented tubal blockage; hydrosalpinx; tubal factor infertility established via HSG, other image-based techniques, and/or laparoscopy; presence of adnexal mass; recurrent pelvic inflammation; endometriosis resistant to conventional therapy; or unexplained infertility. We excluded women with infertility due to abnormalities of ovulation, male factors, endocrine-related disorders, and adhesions due to previous abdominal surgery.

2.2 Sample Collection

Endometrial aspirates and tissues were collected from premenstrual endometrium via D&C and/or laparoscopy ($n = 83$) where available. On the second day of the subject's menstrual cycle, using a sterile Cusco's speculum in the vagina to avoid contact with or contamination from the skin, MB was collected with a 5-ml sterile syringe and subsequently stored in sterile vials containing EDTA.

2.3 Histopathological Studies and Culture

We performed histopathology staining (hematoxylin and eosin [H&E] stain) of endometrial tissue for all subjects suspected of having GTB whose endometrial tissues were available. For histopathological studies, a portion of the endometrial tissue was fixed in 10% formalin and stained with H&E. Caseating granulomas surrounded by epithelioid cells, lymphocytes, plasma cells, and giant cells were considered TB [12]. Mononuclear cells were derived from MB via density gradient centrifugation using histopaque (Sigma-Aldrich, St Louis, MI, USA; Cat. no. 10771) at a 1:1 ratio and dissolved in phosphate-buffered saline (PBS). Cells and homogenized tissue pellets were inoculated with

Löwenstein–Jensen (LJ) culture media (Himedia, Mumbai, India; Cat. no. M162) and incubated at 37 °C in a Bio-Oxygen Demand (BOD) incubator for 8 weeks [13].

2.4 Multiplex Polymerase Chain Reaction

DNA was extracted from clinical specimens (day 2 MB and tissue) using a QIAmp DNA mini kit (Qiagen, Hilden, Germany; Cat. no. 51306). Finely chopped tissue (≤ 20 mg) was dissolved in TE buffer (pH 8) and centrifuged at 10,000 rpm for 10 min. After supernatant was discarded, pellets were dissolved in TE buffer and DNA was isolated using a QIAmp DNA mini kit as per manufacturer's instructions, and 200 ul of MB was used for DNA isolation. Primer details are presented in Table 1. Here, we used M-PCR to target three regions of the MTBC genome: hsp65, DNA J, and IS6110, with amplicon length 165, 365, and 541 bp, respectively [14–16]. PCR amplification was performed in a thermal cycler with an initial cycle of denaturation (5 min. at 95 °C), followed by 40 cycles each of 1 min. at 94 °C, 1 min. at 55 °C, and 2 min. at 72 °C, with a final extension of 10 min. at 72 °C. Each DNA amplification experiment included a positive control (reference strains: *M. tuberculosis H37Rv*-DNA) and a negative control (sterile distilled water). All PCRs were done in 25-μl reaction volumes, and final reagent concentrations were as follows: 1X Dream Taq reaction buffer with 2 mM MgCl$_2$ (Thermo Fisher Scientific, Waltham, MA, USA; Cat. no. EP0701), 250 μM each deoxy ribonucleotide triphosphate (dNTPs), 1 μM each specific primer and 0.25 units Dream Taq DNA Polymerase (Thermo Fisher Scientific; Cat. no. EP0701), and 20–100 ng DNA. PCR was performed using a Biometra-T-Personal 48 Thermal Cycler (Goettingen, Germany). The amplified products were separated by electrophoresis on 2% agarose gel stained with 0.5 μg/ml ethidium bromide and visualized and photographed under an ultraviolet transilluminator. Reference strains of *M. tuberculosis-H37RV* used as controls were obtained from the Tuberculosis Research Centre (TRC), Indian Council of Medical Research, Chennai, India (Fig. 1 in the Electronic Supplementary Material).

2.5 Statistical Analysis

No gold standard diagnostic method is available to confirm a diagnosis of GTB. Here, we consider subjects to be true positive if they are found to be positive for TB via three methods: histopathology and M-PCR from both endometrial tissue and MB ($n = 83$). Subjects who had negative results from all three methodologies were considered "true negative", on this basis we calculated the sensitivity, specificity, negative predictive value and positive predictive value of the methodologies, histopathology and M-PCR, from both endometrial tissue and MB (MedCalc Statistical Software version 16.4.3; Ostend, Belgium; 2016).

3 Results

Study subjects were aged between 28 and 42 years. The clinical characteristics of subjects with suspected GTB ($n = 150$) are presented in Table 2. Primary infertility and menstrual abnormality were the most common characteristics, with 61 (31.3%) subjects having some degree of menstrual disturbance, primarily oligomenorrhea but also amenorrhea or menorrhagia. In total, 106 (54.3%) subjects had unexplained infertility, and 49 (25.1%) had tube-related complications, such as tubal blockage, hydrosalpinx, and tubal factor infertility. Furthermore, 29 (14.8%) subjects had pelvic inflammation, and 11 (5.6%) had endometriosis resistant to conventional therapy. We also observed that nine women had been previously affected by PTB and one subject had been in prolonged contact (6 months) with PTB. Of the 195 subjects clinically suspected as having GTB, 71 (36.4%) tested positive for TB using all three methodologies (histopathological staining and M-PCR, using both tissue and MB). Discordance was evident among the results from the different methodologies: 49 (25.1%) individuals were positive for TB via histopathological staining for M-PCR using tissue 69 (35.4%) and using MB as the source of the sample 66 (33.8%) (Table 2), whereas 46 subjects (23.3%) tested positive using all three methodologies (100% concordance). This suggests that the sensitivity and specificity of

Gene	Primers (5′–3′)	Amplicon (bp)
65 KDa	CTA GGT CGG GAC GGT GAG GCC AGG	165
(*hsp proteins*)	CAT TGC GAA GTG ATT CCT CCG GAT	
DNA J	AAG AGG AAG GAG AGA GGC	365
	GTC GTT GAG GTT GAA CTC	
IS 6110	GTG GGC ATG GTC GCA GAG AT	541
	CTC GAT GCC CTC ACG GTT CA	

Table 1 Primer details of multiplex-polymerase chain reaction for diagnosis of genital tuberculosis [14–16]

Table 2 Clinical characteristics and diagnoses of genital tuberculosis according to diagnostic approach

	Image	Laparoscopy	TB PCR positive	TB PCR positive	TB positive by histopathology from ET	TB positive by culture	TB positive by culture
			MB	ET		ET	MB
Clinical features							
Primary infertility	195	41	66	69	49	7	3
History of PTB	9	–	2	2	1	–	–
Prolonged contact with PTB	1	–	–	–	–	–	–
Menstrual abnormality							
No menstrual abnormality	134	29	37	36	28	2	–
Amenorrhea	3	–	1	2	2	–	–
Oligomenorrhoea	41	11	23	29	18	2	1
Menorrhagia	17	1	5	2	1	3	2
Infertility-related complication							
Unexplained infertility	106	27	38	41	31	2	–
Tube-related complication, e.g., tubal blockage, hydrosalpinx, and tubal factor infertility	49	21	14	12	9	2	2
Recurrent pelvic inflammation	29	–	9	8	6	3	1
Endometriosis resistant to conventional therapy	11	3	4	5	3	–	–

ET endometrial tissue, *MB* menstrual blood, *PCR* polymerase chain reaction, *PTB* pulmonary tuberculosis, *TB* tuberculosis

the methodologies varied greatly. As no gold standard diagnostic methods for GTB yet exist, we considered 46 subjects as being true positive (because they were positive for all methodologies) to compare the sensitivity and specificity of the methods tested.

The sensitivity and specificity of M-PCR using MB was 90.2 and 86.1%, M-PCR using endometrial tissue was 95.8 and 84.3%, and for histopathology staining was 64.8 and 93.2%, respectively. The LJ culture failed to produce acceptable result as only three samples (4.2%) using MB and only seven (9.8%) using endometrial tissue were positive (Table 3).

4 Discussion

Infertility due to MTBC is important to consider in a community with a high prevalence of PTB [2]. TB seems a serious threat for India, as disease prevalence increased from 251 per 100,000 population in 2012 to 651 per 100,000 in 2013 despite a continuous reduction of TB incidence since 1990 [17]. After the lungs, the genitourinary tract is the most common site for TB infection [18] and recent evidence has shown progression of TB infection from the lungs to the genitourinary region [19]. This

Table 3 Sensitivity and specificity of methods used to diagnose genital tuberculosis

	PCR from MB ($n = 83$)	PCR from ET ($n = 83$)	Histopathology from ET ($n = 83$)	Culture from ET ($n = 83$)	Culture from MB ($n = 83$)
Sensitivity	90.2 (78.6–96.7)	95.8 (85.7–99.5)	64.8 (52.5–75.7)	41.8 (32.5–51.6)	40.3 (31.3–49.9)
Specificity	86.1 (79.4–91.3)	84.3 (77.4–89.8)	93.23 (87.5–96.9)	100 (97.1–100)	100 (97.1–100)
Positive predictive value (NPV)	69.7 (62.8–75.8)	66.67 (60.7–72.1)	93.9 (84.6–97.7)	–	–
Negative predictive value (PPV)	77.3 (57.9–89.3)	87.5 (62.8–96.6)	32.43 (24.2–41.8)	–	–

Data are presented as *n* (95% CI)

CI confidence interval, *ET* endometrial tissue, *MB* menstrual blood, *NPV* negative predictive value, *PCR* polymerase chain reaction, *PPV* positive predictive value

observation is alarming in areas where PTB is common, at least in females.

Hence, clinic-based cost-effective, sensitive, and rapid screening is crucial for the diagnosis of GTB where suspected. Reports have addressed the role of TB in subfertility and infertility [18, 19]. GTB invariably affects the fallopian tube, and the endometrium is involved in 50% of cases [20]. The incidence of infertility in GTB varies from 10 to 85%. MTBC in the genitals can cause tubal obstruction, impaired implantation due to endometrial involvement, and ovulatory failure due to ovarian involvement [21]. This clinic-based study demonstrated that 36.4 and 25.1% of subjects with primary infertility clinically suspected of having GTB [results from image-based techniques, or laparoscopy (n=150)] were diagnosed using PCR-based methods and histopathological diagnosis, respectively, from endometrial tissue. The data suggest that image-based diagnosis may overestimate the prevalence of GTB when compared with histopathological or PCR-based diagnosis. A recent report [22] concluded that the prevalence of GTB ranged from 3 to 41% in India and postulated that this range is due to diagnostic dilemma [22]. Currently, the culture-based diagnosis is the gold standard diagnostic approach for PTB, but these methods have severe limitations for EPTB, particularly GTB [8]. It has been anticipated that a significant number of subjects remain undiagnosed because specific non-invasive diagnostic tools are not available. The current invasive procedures, either laparoscopic evidence or D&C for collection of endometrial tissue, seem the most crucial hurdle for GTB diagnosis. Although costly laparoscopic evidence has been reported as being the most sensitive, significant concern surrounds its specificity [8]. PCR using endometrial tissue has been demonstrated as one of the most sensitive diagnostic procedures to detect tubercular involvement in the female genital tract [8].

In the present study, we have, for the first time, reported an alternative sample strategy to overcome the sampling hurdle. We have demonstrated that M-PCR from MB is a sensitive (90.2%), rapid, specific (86.1%), and non-invasive diagnostic tool compared with other methodologies involving endometrial tissue as the sample source (Table 3). The present study also demonstrated that the cultured sample has the lowest sensitivity (40.3%), followed by histopathological staining (67.8%). Histopathological diagnosis of GTB is not specific; GTB can mimic other conditions, including sarcoidosis, syphilis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, and pneumoconiosis [8]. The differences between MB and endometrial tissue as samples in terms of sensitivity and specificity for M-PCR-based assay is almost insignificant (Table 3).

The sensitivity and specificity of our in-house M-PCR from MB was 90.2 and 86.1%, respectively, whereas it was 95.7 and 100% for pulmonary TB using sputum as a sample source [14–16]. We used three amplicons of 165, 365, and 541 base-pairs targeting genes, *hsp65*, *DNAJ*, and IS6110 [14–16]. Genes encoding 65 kDa heatshock protein (HSP), IS 6110 insertion sequences, DNAJ, and 16S rRNA are usually independently used as a target sequence for detection of mycobacterial DNA from both pulmonary and extra-pulmonary clinical samples [19]. Worldwide, multiple well-validated PCR-based tools have been developed to overcome the limitations of conventional methods with great sensitivity, ranging from 77 to >95% [11]. Kulkarni et al. [23] report variations in specificity and sensitivity when using a single target. In this context, our method provides additional confidence with increased sensitivity by introducing multiple targets compared with conventional single target-based nucleic acid amplification tools. This MB-based M-PCR diagnostic approach for GTB may be advantageous for the developing world as it bypasses the costly, painful, and invasive sampling procedure required to collect endometrial tissue. Diagnostic methods involving endometrial tissue obtained via laparoscopy or D&C and other non-specific image-based tools may not be acceptable to people in developing countries, as the cost and pain of these invasive processes remain a significant issue.

Here, our M-PCR-based method using MB provided a non-invasive, rapid diagnostic tool for GTB, an asymptomatic EPTB. It eliminates the invasive procedures required to sample endometrial tissue and their associated pain. The present effort may be crucial as ectopic pregnancy, recurrent miscarriage, tubal abnormality, and unexplained infertility are currently predominant, at least in developing countries such as India. The use of the simple cost-effective method to diagnose GTB in clinic-based screening for women of reproductive age seems advantageous as it allows for early chemotherapeutic intervention. Subsequently, it may protect from irreversible damage that often results in failure to conceive. The main limitation of this study is the sample size, hence further validation with a larger cohort will be necessary.

The method is suitable for non-invasive, cost-effective primary screening for GTB, perhaps before confirmation via endometrial tissue-based histopathological staining.

Author Contributions SKP and BB were involved in the study design and data generation and analysis. AB was involved in study design and analysis. RGC and SC conducted the clinical phenotyping and diagnosis, and CB assisted with analysis and manuscript editing. All authors were actively involved in manuscript preparation.

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