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DETECTION OF ACTIVE FEMALE GENITAL TUBERCULOSIS BY MOLECULAR METHOD

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ABSTRACT

A molecular method for early detection of active female genital tuberculosis from clinical endometrial tissue specimens was developed by modifying single tube nested reverse transcriptase polymerase chain reaction. The reliability of this method was compared with the gold standard culture method (a combination of Mycobacterium Growth Indicator Tube system and Lowenstein Jiensen media) that is a still corner stone on which a definitive diagnosis relies. The viability of *Mycobacterium tuberculosis* (half-life only 2-3 min), excellent yield and purity of isolated total ribonucleic acid from clinical tissue specimens were obtained by using commercially available kits and reagents. The use of special negative control (template without reverse transcriptase enzyme) makes the modified method more reliable for diagnostic purposes, since it checks deoxyribo nucleic acid contamination. Finally, early detection of active female genital tuberculosis is possible by this modified method rather than time consuming culture methods.

KEY WORDS

M.tuberculosis, polymerase chain reaction, 85B antigen, RT-PCR, genital tuberculosis, STN RT-PCR

INTRODUCTION

More than 2 billion people, equal to one-third of the world's population are infected with tuberculosis (TB) bacilli. 1 in 10 people with TB bacilli will become sick with active TB in their lifetime[1]. TB exists in two forms; pulmonary tuberculosis and extra-pulmonary tuberculosis. Genital tuberculosis is one form of extra-pulmonary TB that affects about 12% of patients with pulmonary tuberculosis and represents 15-20% of extra-pulmonary tuberculosis[2]. It is estimated that 5-13 percent of the females presenting in infertility clinics in India have genital TB and majority are in the age group of 20-40 years[3]. Moreover, it is also estimated that at least 11% of the patients lack symptoms and genital TB is often detected in diagnostic

workup of women attending infertility clinics[4]. In genital TB; the fallopian tubes are affected in almost 100% followed by the endometrium in 50%, ovaries in 20%, cervix in 5% and vagina and vulva in <1%[5-6]. There is no way to take the fallopian tube out, but a specimen from the endometrium can be taken to detect genital TB.

Early diagnosis of tuberculosis and initiating optimal treatment would not only enable a cure of an individual patient but will also curb the transmission of infection and disease to others in the community. Conventional diagnostic tests, excluding culture method, are unable to detect active tuberculosis. Culture method is still corner stone on which definitive diagnosis of tuberculosis relies, but it is time consuming due to very slow growth of *Mycobacterium*[7].

Molecular methods like polymerase chain reaction (PCR) are highly specific, sensitive and very fast as compared to the gold standard culture method. BD MGIT (Mycobacteria Growth Indicator Tube) system is a rapid and sensitive method, amongst other culture methods, for early diagnosis of pulmonary and extra-pulmonary TB but for maximum recovery of *Mycobacteria;* a combination of both MGIT and LJ media should be used[8]. False positive cases reported in TB PCR are basically because of contamination of clinical specimens with *M.tuberculosis* DNA product from the PCR laboratory. A special negative control (template RNA without RT enzyme) has been introduced in our assay to check the DNA contamination. Considerable work has already gone into resolving the problem of contamination[9-11]. Therefore, we have focused on the issues of preserving microbial viability in endometrial tissue specimens and STN RT-PCR.

In viable *Mycobacteria*; there is plasmid DNA, 16S rRNA and mRNA while, in dormant stage of *Mycobacteria*; there is DNA and 16S rRNA and in dead stage; there is only DNA present in *Mycobacteria*. We took advantage of the fact that mycobacterial mRNA has an extremely short half-life, averaging only 2-3 minutes[12], so by targeting mycobacterial mRNA by the reverse transcription PCR (RT-PCR) would indicate the presence or absence of recently viable organism. Moreover, the nested RT-PCR would make the procedure more reliable for diagnostic purposes because it can detect even single copy of the gene of MTB; hence it is highly specific and sensitive than RT-PCR. The mRNA coding for the ubiquitous 85B antigen protein is known to be secreted in large quantities from growing *Mycobacteria*[13].

MATERIALS AND METHODS

Clinical specimens

One hundred and twenty five patients, aged 25-45 years, appeared to the infertility clinic (Jaipur Fertility & Microsurgery Research Centre, Jaipur, India) of Mahatma Gandhi Medical College & Hospital, Jaipur, India and were investigated for the infertility due to genital tuberculosis. A protocol for fertility work up included; complete history, clinical laboratory examinations (ESR, MT test, TBIgG & TBIgM, Fertility hormones etc), ultrasonography, hysteroscopy and laparoscopy.

Sixty patients, out of one hundred and twenty five, were suspected on the basis of strong clinical and radiological evidence of genital TB and endometrial biopsy was done on the first day of menstruation. The specimens were taken from the endometrium, especially from both cornual ends, in sterile phosphate buffer saline (PBS) vials and in the RNA*later* RNA stabilization reagent tubes (Qiagen, Germany) respectively for *Mycobacterium* culture and for single tube nested reverse transcription polymerase chain reaction (STN RT-PCR).

Processing of specimens for culture

All the specimens (sixty) were processed in the department of Microbiology of Mahatma Gandhi Medical College & Hospital, Jaipur, India. Each endometrial tissue biopsy (from PBS) was minced as finely as possible and was centrifuged for 20 min at 3000xg. Supernatant was discarded and the pellet was reconstituted with 1ml of sterile phosphate buffer, 0.5 mL of this was added to the BBL

MGIT tubes (from Becton-Dickinson) containing 4ml of modified Middlebrook 7H9 broth base and two drops on the LJ media. Lyophilized MGIT PANTA (containing polymixin B, azlocillin, nalidixic acid, trimethoprim, amphotericin B) was reconstituted with MGIT growth supplement (containing oleic acid, albumin, dextrose, catalase, polyoxyethylene strearate), and 0.8ml of this was added prior to sample inoculation. The inoculated media were incubated at 37 ^{o}C; MGIT tubes were incubated for 6 weeks and LJ media were incubated for 8 weeks. Read tubes daily in BD BACTEC MicroMGIT Fluorescence Reader from next day of incubation. A positive control (an uninoculated MGIT tube containing 5ml of 0.4% sodium sulfite solution) and a negative control (unopened, uninoculated MGIT tube) were also used for correctly interpreting results and to detect possible contaminations.

Processing of specimens for Single-tube Nested Reverse Transcription Polymerase Chain Reaction (STN RT-PCR)

Isolation of total RNA from endometrial tissue specimens

Each endometrial tissue (sixty) biopsy in RNA*later* RNA Stabilization reagent (Qiagen, Germany) was subjected to total RNA isolation using commercially available RNeasy Kit, (Qiagen, Germany) strictly as per the guidelines of the manufacturer[14]. The purity of isolated total RNA was measured by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then the isolated total RNA was stored at -80°C in the ultra low deep freezer for further process of STN RT-PCR.

STN RT-PCR

Only MGIT positive samples were processed by STN RT-PCR for amplification of 216bp region of mRNA coding for 85B antigen with following two sets of primers [*Outer primers*; GAG TAC CTG CAG GTG CCG TCG CCG TC and CCG GGT GTT GTT TGC GAC CAG CTT G] and [*Inner primers*; GAC TTA CAA GTG GGA AAC C and CCG ATC AGG CTA GGC CCC].

Outer reverse transcription (RT) was performed with a volume of 25 µL containing 0.8 µM antisense outer primer, 200 µM dNTP (each), 2 µL 10X Buffer, 1.2 µL MgCl2 (25 mM/1.5 mL), RNase inhibitor (1 U), 2.5 U of MMLV reverse transcriptase, 0.5 U of HK-UNG (all Perkin-Elmer) and 4.5 µL of isolated total RNA. Incubated for 10 min at 50°C to allow the HK-UNG to work and then temperature was raised to 60°C for 30 min to initiate synthesis of the first cDNA strand and then to 94°C for 2 min to inactivate the HK-UNG. Moreover, 40 µL of PCR reaction containing 5 µL 10X Buffer, 3 µL MgCl2, 5 U of r*Tth* DNA polymerase (all Perkin-Elmer), 0.8 µM outer sense primer and PCR grade water (Sigma Co. USA) was used to perform outer PCR. The thermal cycler (Hybaid PCR Express) was programmed for 30 cycles of denaturation at 94°C for 45 seconds, with primer annealing and extension carried out in one step at 70°C for one-five minutes. Nested PCR was then performed using 1µL of outer PCR product, 2.5 µL of 10X Buffer, 1.5 µL MgCl2, 200 µM dNTP (each), 2.5 U of r*Tth* DNA polymerase, inner primers at a concentration of 0.8 µM and remaining PCR grade water to make the final volume of 25 µL. The thermal cycler was programmed for 30 cycles of denaturation at 94°C for 45 seconds, primer annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds, then the reaction mixture was held at 72°C for 10 min. A positive control (H37Rv strain; ATCC 27294) and a negative control (Only PCR grade water) were carried out with the samples. Besides these, a special negative control (all STN PCR steps to isolated total RNA without RT enzyme) was also carried out. Then gel electrophoresis (Bangalore genie) was performed with100bp DNA ladder on 1.5% agarose gel (both from Sigma Co. USA).

RESULTS AND DISCUSSION

Active female genital tuberculosis (TB) is an important cause of infertility. Early diagnosis and treatment of genital TB within reproductive age can improve conception rate significantly. Our results showed that genital TB is 20.8% (26/125) from the patients attending our

infertility center (Jaipur Fertility & Microsurgery Research Center).

Initially, one hundred and twenty five infertile female patients were investigated and out of them; sixty were analyzed to rule out active genital TB using endometrial tissue specimens. Out of 60 specimens, 28.33% (17/60) were positive by BD BACTEC MicroMGIT Fluorescence Reader and 15% (9/60) were positive by LJ media. Positive and negative controls (MGIT tubes) indicated that there was no contamination during the culture. The MicroMGIT positive specimens (Seventeen), processed by modified STN RT-PCR, were amplified the region of mRNA coding for 85B protein at 216bp in agarose gel electrophoresis with sharp bands [Fig. 1 (A) and (B)].

Fig. 1 A

M NC1 PC S1 NC2 S2 S3 S4 S5 S6 S7 S8 S9

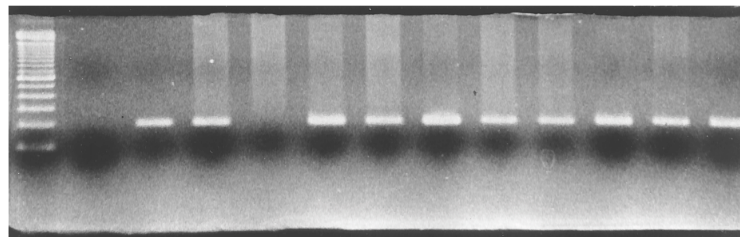

Fig.1. A. Gel electrophoresis of STN RT-PCR products (sample-1 to 9): Lane 1, a 100 bp Marker (M); Lane 2, no template negative control (NC1); Lane 3, Positive Control (PC)-a 216 bp amplification product of STN RT-PCR performed with RNA extracted from *M.tuberculosis* H37Rv strain; Lane 4, a 216 bp amplification product of sample-1 (S1); Lane 5, a special negative control- template without RT enzyme (NC2); Lane 6, a 216 bp amplification product of sample-2 (S2); Lane 7, a 216 bp amplification product of sample-3 (S3); Lane 8, a 216 bp amplification product of sample-4 (S4); Lane 9, a 216 bp amplification product of sample-5 (S5); Lane 10, a 216 bp amplification product of sample-6 (S6); Lane 11, a 216 bp amplification product of sample-7 (S7); Lane 12, a 216 bp amplification product of sample-8 (S8); Lane 13, a 216 bp amplification product of sample-9 (S9).

Fig.1 B

M NC1 PC S10 NC2 S11 S12 S13 S14 S15 S16 S17

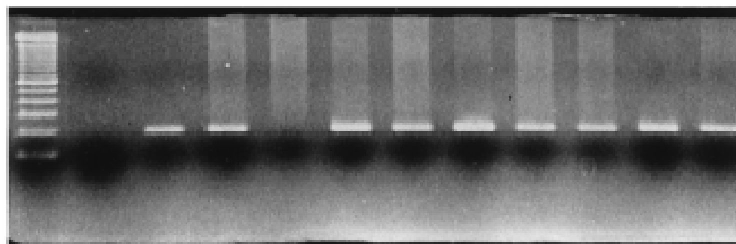

Fig.1. B. Gel electrophoresis of STN RT-PCR products (sample-10 to 17): Lane 1, a 100 bp Marker (M); Lane 2, no template negative control (NC1); Lane 3, Positive Control (PC)-a 216 bp amplification product of STN RT-PCR performed with RNA extracted from *M.tuberculosis* H37Rv strain; Lane 4, a 216 bp amplification product of sample-10 (S10); Lane 5, a special negative control- template without RT enzyme (NC2); Lane 6, a 216 bp

amplification product of sample-11 (S11); Lane 7, a 216 bp amplification product of sample-12 (S12); Lane 8, a 216 bp amplification product of sample-13 (S13); Lane 9, a 216 bp amplification product of sample-14 (S14); Lane 10, a 216 bp amplification product of sample-15 (S15); Lane 11, a 216 bp amplification product of sample-16 (S16); Lane 12, a 216 bp amplification product of sample-17 (S17).

The special negative control (NC2) was not amplified, which showed that there is no contamination of DNA during the whole procedure (including isolation of total RNA).

Culture of *Mycobacterium tuberculosis* (MTB) remains gold standard for the diagnosis of active genital TB. The sensitivity of traditional culture methods like conventional LJ media culture is quite low, especially in the specimens containing small number of organisms. A variety of manual and automated systems have been developed specifically to reduce the time to detect and identify *Mycobacteria* in clinical specimens[15]. In our assay, Mycobacteria Growth Indicator Tube (MGIT) and culture on LJ media were used for maximum recovery; 28.33% (17/60) were positive by BD BACTEC MicroMGIT Fluorescence reader and 15% (9/60) were positive by LJ media. The maximum recovery was 43.33% (26/60) from both culture methods. Positive control (an uninoculated MGIT tube containing 5ml of 0.4% sodium sulfite solution) and negative control tubes (unopened, uninoculated MGIT tube) were also used to detect any contamination during culture.

Due to advent of molecular biological techniques like PCR, it is now possible to detect viable MTB with increased specificity, sensitivity and rapidity rather than time consuming culture methods. Other molecular biological techniques; based on DNA and ribosomal RNA (rRNA) are not suitable to diagnose active TB because they can diagnose dead and dormant stage *Mycobacteria* respectively. Since mRNA is present only in active tubercle bacilli, so mRNA based PCR would be the method of choice for the diagnosis of active genital TB. By our modified STN RT-PCR method, all seventeen (MGIT positive) were positive with the positive control; *M.tuberculosis* H37Rv strain [ATCC27294].

Mycobacterial mRNA has an extremely short half-life, averaging only 2-3 minutes[12], so by preserving the specimens in RNA*later* RNA Stabilization reagent (Qiagen, Germany) would provide the required template for RT-PCR that indicates the presence or absence of recently viable organism. Moreover, total RNA were isolated using commercially available kits (RNeasy Kit, Qiagen, Germany) to get better yield and excellent purity required to get better and accurate results for diagnostic purposes. The two commonly used reverse transcriptase enzymes (RTs) are avian myeloblastosis virus reverse transcriptase (AMV RT) and Molony murine leukemia virus reverse transcriptase (MMLV RT). AMV RT is more robust than MMLV RT[16] retains significant polymerization activity up to $55^{0}C$[17] and can help eliminate problems associated with RNA secondary structure. In contrast MMLV RT has significantly less RNase H activity than AMV RT[18]. As even reduced RNase H activity can interfere with the synthesis of long amplicons[19], MMLV RT may be a better choice if the aim of the experiment is the amplification of full length cDNA molecules. In our assay, we have used MMLV RT. Nested RT-PCR is more sensitive because it can detect even single copy of DNA available in the clinical sample; hence it is more valuable in case of diagnostics. r*Tth* DNA polymerase provides excellent amplification in nested PCR rather than T*aq* DNA polymerase.

Contamination is the main disadvantage with molecular diagnosis of infectious diseases. Single tube method (STN RT-PCR) prevents the carryover contaminations because the whole reaction is completed in a single tube. We have used a negative control (only PCR grade water) and a special negative control (isolated total RNA i.e. template RNA without RT enzyme) to check DNA contaminations (by special negative control; NC2) as well as any contamination in the reagents used during PCR. The special negative control should not be amplified because there is no RT enzyme, so the template couldn't convert into DNA that

can be amplified by PCR or by nested PCR. Moreover, we have used UNG for further protection against contamination. The use of UNG would normally be impossible with a nested format because its use in the second stage of the nested PCR would eliminate the amplified target generated in the first stage. Since our STN RT-PCR procedure is performed in a single tube and no transfers are necessary, the UNG can be inactivated prior to the initiation of amplification. Although UNG rapidly removes uracil from DNA, the target in this assay; mRNA remains unaffected.
The bands of the amplified products on the agarose gel revealed very sharp, on 216bp with the positive control, to detect the positive and negative specimens clearly. The use of commercial kits and reagents for maintaining the viability of mycobacteria, isolation of RNA and modification in the protocols of STN RT PCR from earlier described protocols[20], improved the results as seen on gel in the form of sharp bands.

CONCLUSION

This modified molecular method (STN RT-PCR) can be used for the diagnosis of active genital TB from clinical endometrial tissue specimens. The only drawback of this modified STN RT-PCR method is its expensiveness; due to the cost of infrastructure, use of commercial kits, reagents to avoid contaminations and to preserve the viability of MTB.

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