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A STUDY ON THE USE OF MENSTRUAL BLOOD SAMPLING IN THE DETECTION OF FEMALE GENITAL TUBERCULOSIS BY NUCLEIC ACID AMPLIFICATION TECHNIQUE (NAAT)

General Medicine

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ABSTRACT

Objectives: To assess the prevalence of multidrug resistant tuberculosis (MDR-TB) and rifampicin resistance in females of fertile age group suffering from genital tuberculosis using menstrual blood (MB), through nucleic acid amplification technique (NAAT).**Methods:** A prospective observational study was conducted for a period of 23 months which included 50 women of clinically suspected genital tuberculosis of childbearing age Day 1 or Day 2 MB of all females of child bearing age was taken. The specimen were subjected to AFB testing by ZN staining and NAAT by Truenat for Mycobacterium tuberculosis (MTB) DNA detection and Rifampicin resistance. The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0. **Results:** The mean age of study subjects were 26.6 ± 6 years. MB was positive for AFB Stain (ZN Stain) in 50% cases but MTB DNA was detected through Truenat in 1 only out of 50 patients. MDR -TB suspicion on the basis of ATT response was present in 7 out of 50 patients(14%) but none of the patients showed Rifampicin resistance on NAAT technique.**Conclusion:** We performed this study as a sort of Pilot project, which used Menstrual blood as a sample, since it is the most non-invasive sampling and tried to use new technique of Truenat for MTB detection and drug resistance characterisation. The negative findings of the study must be interpreted in consideration of limitations such as small sample size, relatively newer technique and sampling method.

KEYWORDS

Menstrual blood, multidrug resistant tuberculosis, rifampicin resistance

INTRODUCTION

Female genital tuberculosis (FGTB) is a major public health problem in India. It is a primary cause of female infertility with incidence varying from 1% in USA, 21.1% in south Africa and 1-19% in India.[1,2] Primarily it present at a young age of 20-40 years rather than perimenopausal age group.[3]

The diagnosis remains a challenge despite availability of various diagnostic techniques which may be due to mild or no symptoms.[4] Thus, FGTB requires a systematic approach and investigations after a clinical suspicion.[5]

Among the recent techniques, nucleic acid amplification technique (NAAT) has been frequently applied at tertiary care setups. It is based on the principle of identification of nucleic acid of the etiological organism from a variety of tissue samples[6]. It shows good accuracy, rapid turnaround time, thus limiting the false negatives and loss to follow up of patients. Additionally as an advantage, it can identify *Mycobacterium tuberculosis (MTB)* genome mutations which are identified in drug resistance.[7,8]

Among the various techniques of NAAT, the Gene Xpert MTB/Rif test is a cartridge-based fully automated NAAT for *tuberculosis* case detection and rifampicin resistance testing that has been endorsed by WHO worldwide.[9] WHO suggests to use it for the initial diagnosis of MDR-TB in suspected individuals and in the followup of smear negative specimens.[10]

Sharma JB et al.,[11] (2016), reported that Gene Xpert has almost 100% sensitivity and 100% specificity for FGTB. Sharma JB et al (2017),[12] in another study evaluated 6 patients presenting with tubo-ovarian mass or infertility with MDR FGTB where Gene Xpert on endometrial biopsy or sputum was positive in 5 (83.3%) patients, thus concluding that Gene Xpert should be sought for early diagnosis of MDR FGTB. With an early and prompt diagnosis of MDR TB cases, the patients can be managed with multidrug therapy and limited surgeries in the form of drainage of persistent pelvic or tubo-ovarian abscess (as recommended by the American Thoracic Society),[13] to prevent complications such as infertility, ectopic pregnancy and miscarriage.[14] The most common sampling used for the definitive diagnosis of FGTB is endometrial sampling since it is involved in most of the cases and is easily available for sampling. However, a noninvasive way of getting samples for tests is always sought of. One such material, when available, could be menstrual blood (MB).[15,16] MB is a simple, non invasive sample that is useful many times especially in unmarried girls or those who are not willing for invasive tests.[17,18]

The collection of MB can be done on day 1-2 of menstruation from vagina. One of the studies report 10% positivity for *M. tuberculosis* in MB samples.[19] For MB, Acid Fast Bacilli (AFB) culture has also been suggested, however it suffers from low sensitivity.[20,21]

Literature review shows that there is paucity of data on the detection of MDR TB in Indian population with FGTB by using menstrual blood, and it is hypothesized that if a highly sensitive and specific test such as NAAT is applied on the menstrual blood, it may help to detect the cases of FGTB as well as prevalence of MDR TB in a non-invasive manner. Hence this study was undertaken, where we tried to assess the prevalence of MDR TB in females of fertile age group suffering from genital tuberculosis using MB, through newer NAAT diagnostic methods.

METHODS

A prospective observational study was conducted at the Department of Tuberculosis and Respiratory Diseases in association with department of obstetrics and gynecology for a period of 23 months from September 2018 to august 2020. This study included 50 women of clinically suspected genital tuberculosis of child bearing age who attended the clinic OPD during the study period. Pregnant women, patients having bleeding disorders, carcinoma or cervix,uteri or other organ, and Postmenopausal females were excluded. The study was approved by the institutional ethical committee.

The sample size calculation was based on the study of Zahoor D et al,[22]

who observed that prevalence of female genitourinary tuberculosis was 6.73%. Taking this value as reference, the minimum required sample size with 7% margin of error and 5% level of significance is 50 patients. So total sample size taken was 50.

Formula used is:-

$N \geq (p(1-p))/(ME/z_\alpha)^2$

Where Z_α is value of Z at two sided alpha error of 5%, ME is margin of error and p is prevalence rate.

CALCULATIONS:-

$n>=((.0673*(1-.0673))/(.07/1.96)^2=49.21=50$(approx.)

After informing the patients about the study, a written informed consent was obtained from all the patients. A complete clinical history and examination, including genital examination as per standard practice was done. They were also subjected to routine investigations such as sputum for AFB as per RNTCP protocol,[23] complete blood count, Serum glutamic-pyruvic transaminase (SGPT)/(ALT), Alkaline phosphatase, serum bilirubin, Fasting and 2 hours after oral glucose test and chest X-ray PA view. Urine pregnancy test was also done. Other radiological views and investigations if required were done. An ultrasound examination of abdomen was done. If required a Trans-vaginal ultrasound (TVS) examination of the genital system was done. In females of childbearing age group along with amenorrhea, after ruling out pregnancy using urine pregnancy test, an endometrial biopsy was taken.

Day 1 or Day 2 Menstrual Blood of all females of child bearing age was taken. The specimen were subjected to AFB testing by ZN staining and NAAT by Truenat for Mycobacterium tuberculosis detection and Rifampicin resistance.

Trunat™ MTB Plus

Truenat™ MTB Plus (REF 601130005 / 601130020) is a chip-based Real Time Polymerase Chain Reaction (PCR) test for the semiquantitative detection and diagnosis of *Mycobacterium tuberculosis* (MTB) in human pulmonary (sputum/non-sputum) and EPTB specimen and aids in the diagnosis of infection with MTB. Truenat™ MTB Plus runs on the Truelab™ Real Time Quantitative micro PCR analyzers.[24]

Trunat MTB-RIF Dx Chip-based Real Time PCR Test for Rifampicin Resistant *Mycobacterium tuberculosis*

Truenat™ MTB-RIF Dx (REF 601210005 / 601210020/601210050) is a chip-based Real Time Polymerase Chain Reaction (PCR) test for the detection of Rifampicin resistance in *Mycobacterium tuberculosis* (MTB) in Truenat™ MTB/MTB Plus positive human specimen and aids in the diagnosis of MDR-TB. This test detects the presence of major mutations (SNPs) in the MTB genome that are known to cause resistance to rifampicin. Truenat™ MTB-RIF Dx runs on the Truelab™ Real Time Quantitative micro PCR Analyzers. This is a follow on test, to be performed only on the extracted DNA from Truenat™ MTB/MTB Plus positive sample.[25]

After a suspected or confirmed diagnosis, the patients were started with standard anti-tubercular treatment (ATT). The response to the treatment was observed in the initial 2 months of therapy and those who failed to respond were considered cases of multidrug resistant TB.

STATISTICAL ANALYSIS

Categorical variables were presented in number and percentage (%) and continuous variables were presented as mean ± SD and median. Qualitative variables were compared using Fisher's Exact test. P value <.05 was considered as statistically significant. The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

RESULTS

In present study, mean age of study subjects was 26.6 ± 6 years with median (IQR) of 25(22-29.75). The clinical features among the study patients comprised of Infertility (28%), lower abdomen pain (28%), weight loss (24%), irregular menstruation (18%), hypomenorrhea (18%), fever (16.00%), oligomenorrhea (14.00%). Comorbidities were present only in 6 out of 50 patients (12%) which included hypertension, diabetic mellitus and hypothyroidism.(Table 1)

Table 1:-Distribution of baseline characteristics.

Variables	Frequency	Percentage
Age(years)		
18-20	8	16.00%
21-30	30	60.00%
31-40	12	24.00%
Mean ± SD	26.6 ± 6	
Median(25th-75th percentile)	25(22-29.75)	
Range	18-40	
Clinical features suggestive of genital tuberculosis		
Lower abdomen pain	14	28.00%
Fever	8	16.00%
Infertility	14	28.00%
Oligomenorrhea	7	14.00%
Irregular menstruation	9	18.00%
Per vaginal discharge	4	8.00%
Hypomenorrhea	9	18.00%
Inter menstrual bleeding	2	4.00%
Menorrhagia	2	4.00%
Weight loss	12	24.00%
Comorbidities		
Absent	44	88.00%
Present	6	12.00%
Type of co-morbidities		
Hypertension	3	6.00%
Hypothyroidism	3	6.00%
Diabetes mellitus	1	2.00%

In present study, MDR -TB suspicion on the basis of ATT response was present in only 7 out of 50 patients(14%).

For the supportive data, Endometrial tissue samples were used in 11 (22%) patients for MTB DNA detection through Truenat. However, by Truenat, none of these eleven samples showed MTB DNA. Out of 50 patients, one (2%) patient was found to be culture negative for Mycobacterium tuberculosis (Suspected case of Mycobacterium Other Than Tuberculosis - MOTT), and that patient showed improvement on MOTT treatment. Bilateral fimbrial block on hysterosalpingography was found to be positive only in one (2%) patient. (Table 2)

Table 2:-Distribution of Supportive data for MDR TB diagnosis in study subjects

Supportive data for MDR TB diagnosis	Frequency	Percentage
Absent	37	74.00%
Culture negative for Mycobacterium tuberculosis (Suspected case of Mycobacterium Other Than Tuberculosis - MOTT), Improvement seen on MOTT treatment	1	2.00%
Endometrial tissue sample Real time PCR for MTB DNA - Not detected	11	22.00%
Hysterosalpingography - Bilateral Fimbrial block	1	2.00%

MB was positive for AFB Stain (ZN Stain) in 50% cases and MTB DNA was detected through Truenat in 1 only out of 50 patients. None of the patients showed Rifampicin resistance on NAAT technique.

No significant difference was seen in the distribution of ATT response between Menstrual blood AFB positive Stain and AFB negative stain (p value >.05). Proportion of Responders was 92% in negative AFB stains while 80% in positive AFB stains. Proportion of Non responders was 8% in negative AFB stains while 20% in positive AFB stain, with no significant difference between them.(Table 3)

Table 3 :- Comparison of ATT response between Menstrual blood AFB positive stains and AFB negative stains

ATT response	Negative (n=25)	Positive (n=25)	Total	P value	Test performed
Non responders	2 (8%)	5 (20%)	7 (14%)	0.417	Fisher Exact test
Responders	23 (92%)	20 (80%)	43 (86%)		

DISCUSSION

TB has been growing in India and causing a significant effect on the fertility of the females. The recent advances in the diagnostic protocols has led to the increasing use of Nucleic acid amplification tests which

is a PCR based assay to assess the nucleic acids of the organisms. It shows a higher level of detection even with a minor level of infection.

In this observational cross-sectional study, we included 50 cases of clinically suspected genital tuberculosis of child bearing age and determined the Mycobacterium Tuberculosis infection and Rifampicin resistance by the use of Truenat Duo machine which plays an integral part by different modes like- MTB, MTB PLUS, and MTB-RIF Dx. The unique characteristic of the study was that we used menstrual blood as sample for testing since it is one of the most non-invasive way. The mean age of the study population was 26.6 ± 6 years with age ranging from 18-40 years. The women had a plethora of clinical features which helped us to suspect TB such as infertility (28%), lower abdomen pain (28%), followed by weight loss (24.00%), irregular menstruation (18%), hypomenorrhea (18%), fever (16.00%) and oligomenorrhea (14.00%). Among the co-morbidities, in 6.00% of patients, hypertension and hypothyroidism was present and 1 patient had diabetes.

Among the other studies, Rozati et al, reported that the median age of the subjects was 28 (range 18-39) years. Symptoms included pelvic pain and irregular menstrual bleeding, scanty menstruation and amenorrhea. In a case study of 40 year old female patient with secondary vulval TB masquerading as a tumour by Arakeri et al, symptoms of women was Vulval growth as multiple nodules (with sinuses), yellowish-white discharge. In another case study by Agrawal et al, including a 26 year old female with cervical tuberculosis mimicking carcinoma, symptoms included Abdominal pain, vaginal discharge, post-coital bleeding, intermenstrual bleeding, and loss of weight.

In the present study, on assessing all the samples by Truenat, menstrual blood Real time PCR for MTB DNA was detected in only 1 out of 50 patients. The detection rate of MTB DNA was not substantial as per the Truenat.

The low detection as seen in our study can be due to the presence of various blood Inhibitors such as bacteria and human DNA in MB may prevent MTB detection through NAAT[29] ; Normal vaginal flora "Mycobacterium Smegmatus" or other species of Mycobacterium may also be present in menstrual blood sample which may come as AFB ZN Positive results but not detected on NAAT.[30] False-negative results may occur due to the inefficient extraction and amplification of the DNA because of low mycobacterial numbers.[31]

In our study, endometrial tissue samples were also used in eleven patients for MTB DNA detection through Truenat. However, by Truenat, none of these eleven samples showed MTB DNA. Out of 50 patients, one patient was found to be culture negative for Mycobacterium tuberculosis (Suspected case of Mycobacterium Other Than Tuberculosis - MOTT), Patient showed improvement on MOTT treatment. Bilateral fimbrial block on hysterosalpingography was found to be only in one out of 50 patients.

Compared to the Truenat, Sharma JB et al (2017),[11] found GeneXpert a better diagnostic method on endometrial biopsy where 5/6 patients who presented with tubo-ovarian mass or infertility were diagnosed. Chaubey et al, used *hsp65* Nested PCR for FGTB detection using MB and reported a detection rate of 49.% which was much higher than our study. In study performed by Bajpai et al, including 227 patients suspected of suffering from GTB, total 126 samples were found to be positive by PCR. In this study, by Zahoor et al, GeneXpert was done among 69 patients, out of which 5 were positive, two of the samples were positive on both culture and GeneXpert.,

Thus, the detection of mycobacterium tuberculosis on menstrual blood must be done with caution. Though it is a non-invasive method, its detection rate is much less in menstrual blood as compared to other methods.

RIF resistance detection by Truenat

In present study, MDR -TB suspicion on the basis of ATT response was present in only 7 out of 50 patients. However, by Truenat, none of the cases showed Rifampicin resistance. This may be due to various reasons (1) incorrect performance of the technique from pre-testing phase to post-testing phase; (2) the presence of rare mutations in the target genome where the primer binds (3) specimen contamination thereby preventing amplifications and (4) high sensitivity but low specificity of the assay.[22,38]

Although we did not detect any case of RIF resistance with Truenat, Sharma JB et al,[11] (2017) in an observational study assessed six patients who presented with tubo-ovarian mass or infertility with multi drug resistant MDR FGTB, where GeneXpert was able to detect 5 cases of RIF resistance.

CONCLUSION

We performed this study as a sort of Pilot project, which used Menstrual blood as a sample, since it is the most non-invasive sampling and tried to use new technique of Truenat for MTB detection and drug resistance characterisation. In view of the various limitations such as small sample size, relatively newer technique and sampling method, the study results must be interpreted with caution and future long term studies are warranted to determine the ways to eliminate inhibitors in the blood and increase its sensitivity and specificity.

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