Revolutionizing chronic endometritis diagnosis: real-time polymerase chain reaction unveils microbial pathogens in Indian women with abnormal bleeding and reproductive challenges

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BACKGROUND: This study aimed to assess the utility of real-time-polymerase chain reaction (PCR) for diagnosing chronic endometritis (CE) by targeting 11 prevalent pathogens and to compare the outcomes with conventional culture-based diagnosis.

STUDY DESIGN: A retrospective analysis was conducted on 500 patients with clinical conditions such as abnormal bleeding, in vitro fertilization failure, recurrent implantation failure, recurrent miscarriage, and recurrent pregnancy loss. The prevalence of 11 key pathogens associated with CE was evaluated in endometrial biopsy samples.

RESULTS: In our study, PCR identified 318 cases (63.6%) positive for at least one of the 11 investigated pathogens, while culture-based methods detected 115 cases (23%). Predominant pathogens detected by PCR included *Enterococcus faecalis* (*E. faecalis*) (19%), *Escherichia coli* (*E. coli*) (6.8%), *Staphylococcus aureus* (*S. aureus*) (9%), *Mycoplasma hominis* (5%), *Mycoplasma genitalium* (6.2%), *Streptococcus agalactiae* (*S. agalactiae*) (4.2%), *Ureaplasma urealyticum* (4%), nontuberculous *Mycobacterium* (5.2%), *Mycobacterium tuberculosis* (1.2%), *Neisseria gonorrhoeae* (0.6%), and *Chlamydia trachomatis* (2.4%). Standard culture methods identified *E. faecalis* (10.8%), *S. aureus* (6.2%), *E. coli* (3.8%), and *S. agalactiae* (2.2%).

CONCLUSION: The DICE panel proves itself as a swift, precise, and cost-effective diagnostic tool for detecting both culturable and nonculturable endometrial pathogens in CE. Demonstrating superiority, the Molecular method outshines microbial culture, ensuring accurate and sensitive detection of CE-associated pathogens, harmonizing seamlessly with histology and hysteroscopy findings.

Key words: chronic endometritis, Endometrial tuberculosis, microbial culture, polymerase chain reaction

Introduction

Chronic endometritis (CE), marked by persistent inflammation of the endometrial mucosa due to bacterial infections, leads to silent infertility in reproductive-age females. Diagnostic challenges arise as CE lacks conventional clinical symptoms.[1] Oocyte fertilization and embryo implantation are hindered by endometrial inflammation, contributing to recurrent implantation failure (RIF). Standard diagnostic methods involve

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Tweetable statement: This study reports on the efficacy of Molecular Diagnosis of Chronic Endometritis in Indian CE patients.The study analyzed 500 endometrial biopsy samples from infertile and fertile women with abnormal bleeding or reproductive failures, using molecular diagnostics to identify microorganisms associated with chronic endometritis.Among the 500 patient samples analyzed, 318 (63.6%) patients shown positive for at least one pathogen by molecular method whereas 115 (23%) by culture methods.In patients experiencing abnormal bleeding: 33 out of 116 samples (28.4%) tested positive using PCR. 10 out of 116 samples (8.6%) tested positive using culture methods.In patients with recurrent implantation failure: 169 out of 264 samples (64.1%) tested positive using PCR. 56 out of 203 samples (27.6%) tested positive using culture methods.In patients with recurrent pregnancy loss: 116 out of 181 samples (64.1%) tested positive using PCR. A total of 49 out of 181 samples (27.1%) tested positive using culture methods.

Ethical statement: All procedures described were reviewed and approved by the Institutional Review Board of LifeCell Diagnostics ethics committee in Chennai, India (LC-IEC/23/0-06).

Consent for publication: Not applicable.

Data availability: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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Why was this study conducted?

The technique employed still affects the diagnosis of chronic endometritis. The purpose of this study is to evaluate the most common pathogens among Indian women with Chronic Endometritis conditions and to develop and implement a new molecular approach for the diagnosis of chronic endometritis, including MTB and NTM.

Key findings?

In patients who are infertile but have no symptoms and those who have abnormal bleeding conditions, the use of RT-PCR can help with diagnosis and treatment of chronic endometritis.

What does this add to what known?

To improve the effectiveness of treatment, pathogen-specific target therapy for these phantom endometrial complications could be guided by RT-PCR, which can identify bacterial pathogens that cause chronic endometritis but may be difficult to identify using culture methods.

endometrial biopsies, hysteroscopy, and histology, focusing on plasma cell detection and subjective observations.[2]

Women experiencing infertility, recurrent implantation failure (RIF), or recurrent pregnancy loss (RPL) have a higher susceptibility to developing CE.[3–5] Primary causative organisms include Enterobacteriaceae, Enterococcus, Streptococcus, Staphylococcus, Mycoplasma, and Ureaplasma. Despite its potential asymptomatic nature, CE leads to recurrent miscarriages and implantation failures in up to 40% of infertile individuals. Sexually transmitted diseases like *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) elevate the risk of pelvic inflammatory disease, contributing to ectopic pregnancies and tubal factor infertility. CE's complex etiology involves non-infectious factors, but recent research emphasizes bacterial involvement.[6–9]

Alterations in the endometrial microbiome contribute to CE pathogenesis, with a higher risk associated with sexually transmitted diseases. Limited information on CE from India speculates a global prevalence of up to 10% in reproductive-age women. A history of gynecologic infections or multiple pregnancies increases the likelihood of CE.[10]

The culture-positive rate for CE varies (30%−50%), with anaerobic bacteria prevalent. It's crucial to note that not all CE cases stem from bacterial infections, indicating other contributing factors. Estimating CE prevalence proves challenging due to the absence of distinctive clinical manifestations. Reported prevalence in infertile patients ranges from 2.8% to 39.0%, reaching higher percentages in cases of repeated implantation failure or unexplained recurrent miscarriage. Inconsistent results arise when employing three traditional diagnostic methods on the same individuals.[11–13]

Advancements in diagnosing and treating CE involve identifying and characterizing pathogenic microbes. Molecular diagnosis using polymerase chain reaction (PCR), especially real-time (RT)-PCR, plays a crucial role in clinical diagnostics, offering accurate and timely results. This study aims to compare molecular and microbial culture methods' outcomes across clinical conditions, assessing the prevalence of 11 specific pathogenic bacteria causing CE before and after antibiotic therapy.

Materials and methods

Study participants and specimen collection

Patients displayed any one or more clinical manifestations such as pelvic pain, vaginal discharge, dyspareunia, abnormal uterine bleeding, RPL, recurrent implantation failure or post in vitro fertilization (IVF) treatment suspected for endometritis were included in this study. We received 500 EB samples from patients with indications of endometritis diagnosed primarily by hysteroscopy evaluation during the period of November 2022 to October 2023. Clinicians collect the EB samples divided into two parts for histology (10% neutral buffered formalin and microbial culture and/or DICE panel (saline) containers under sterile conditions for further studies were enrolled in this study.

The endometrial tissue samples were collected aseptically as per standard protocol from various medical centers around India were blindly sent to Life-Cell Diagnostics for the histology and DICE panel test to screen 11 major pathogens including 9 CE pathogen[1] with tuberculosis (TB) and Non Tuberculous Myobacterium (NTM) or *Mycobacterium* other than TB as a diagnostic service with informed consent. For the correlation of our study purpose, all the samples were cultured simultaneously irrespective of clinician's request. Control groups (n=50) were included who had no past history of Endometritis as per the Moreno et al., study.

Inclusion criteria: Patients with abnormal bleeding or Reproductive failure cases under treatment or confirmed diagnosis of CE by conventional methods with acceptance for informed consent were included in this study.

Diagnostic criteria: CE is diagnosed based on the presence of inflammation in the endometrial lining persisting for an extended period, typically more than 6 weeks. This diagnosis is typically confirmed through histological examination of endometrial tissue samples obtained via biopsy, revealing the presence of plasma cells within the endometrial stroma.[1]

RIF is diagnosed after a certain number of failed euploid embryo transfers, commonly defined as three to six unsuccessful IVF cycles.[38]

Exclusion criteria: Hysteroscopically negative for endometritis, clinically undiagnosed as endometritis condition, patient with neoplasia or precancerous hyperplasia conditions, samples sent for DICE panel other than endometrial biopsy specimens such as endocervical

TABLE 1
Details of the primer sequences

S. No	Name of organism	Target gene	Primer sequence	References
1	*Escherichia coli*	gadE	F: TGCCCCATAAGAATTCACAA R: GTGACGATGTCGCTCATACG	Tillman et al.[14]
2	*Enterococcus faecalis*	16S rRNA	F: CGCTTCTTTCCTCCCCGAGT R: GCCATGCGGCATAAACTG	Ryu et al.[15]
3	*Neisseria gonorrhoeae*	PorA	F: AACGCCACGACGGTATGC R: CCGCTGAAACCGGAAAATC	Fukumoto et al.[16]
4	*Streptococcus agalactiae*	Sip	F: ATCCTGAGACAACACTGACA R: TTGCTGGTGTTTCTATTTTCA	Bergseng et al.[17]
5	*Chlamydia trachomatis*	23S rRNA	F: AACGACATTTCTTGCTGCAAAG R: TCAGGACATTTTGCGGATAGG	Sato et al.[16]
6	*Staphylococcus aureus*	Coa	F: GTAGATTGGGCAATTACATTTTGGAGG R: CGCATCTGCTTTGTTATCCCATGTA	Sabet et al.[18]
7	*Mycoplasma genitalium*	G-37T	F: GAGAAATACCTTGATGGTCAGCAA R: GTTAATATCATATAAAGCTCTACCGTTGTTATC	Jensen et al.[19]
8	*Mycoplasma hominis*	yidC	F: TCACTAAACCGGGTATTTTCTAACAA R: TTGGCATATATTGCGATAGTGCTT	Férandon et al.[20]
9	*Ureaplasma urealyticum*	ure	F: ATCGACGTTGCCCAAGGGGA R: TTAGCACCAACATAAGGAGCTAAATC	Cao et al.[21]
10	GAPDH	Human	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA	Dahn et al.[22]

exudates/scrapes, endometrial wash solution, menstrual blood and refusal to provide informed consent were excluded.

All women signed an informed consent form, agreeing to a comparison of procedures used for regular CE diagnosis vs DICE panel test. Furthermore, retrospective analysis of the samples submitted for diagnostic purposes was used in our research study, which had no impact on the patient. The LifeCell Diagnostics ethics committee (IRB), Chennai, India, approved the study under the LC-IEC/23/0-06 code.

Microbial culture: Endometrial samples were processed according to the current standard for microbiological culture using a separate test to detect the presence of the most prevalent CE pathogens. In particular, to detect *C. trachomatis*, *N. gonorrhoeae*, *Ureaplasma urealyticum* (*U. urealyticum*), *Ureaplasma parvum*, *and Mycoplasma hominis* (*M. hominis*) which are non-cultivable strains recoverable from the genital tract processed using in-house RT-PCR method only.

To detect the presence of cultivable microorganisms *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*) and other Gram-positive and Gram-negative pathogens, the endometrial samples were inoculated onto culture media following enrichment in brain heart infusion medium as per standard microbiological methods. Consequently, the growth of the bacteria indicated above >10,000 colony-forming units was considered positive and isolated from the culture. According to microbiology guidelines, the presence of Staphylococcus epidermidis was considered to indicate contamination.

Molecular diagnosis: Endometrial tissue samples were analyzed for the identification of CE pathogens by RT-PCR using specific primers (Table 1) for the 9 most common bacteria responsible for causing endometritis such as *C. trachomatis*, *N. gonorrhoeae*, *E. faecalis*, *E. coli*, *S. agalactiae*, *Mycoplasma genitalium*, *M. hominis*, *U. urealyticum*, *S. aureus. In addition*, *PCR Probes specific to Mycobacterium tuberculosis complex* (*M. tuberculosis*) *M. tuberculosis and Mycobacterium microti*, (*FAM*) *whereas nontuberculous mycobacterium* (*NTM*) (*VIC*) *includes—Mycobacterium abscessus*, *Mycobacterium mucogenicum*, *Mycobacterium avium*, *Mycobacterium nonchromogenicum*, *Mycobacterium celatum*, *Mycobacterium peregrinum*, *Mycobacterium chelonae*, *Mycobacterium scrofulaceum*, *Mycobacterium fortuitum*, *Mycobacterium shimoidei*, *Mycobacterium gordonae*, *Mycobacterium szulgai*, *Mycobacterium intracellulare*, *Mycobacterium terrae*, *Mycobacterium kansasii reported to cause* tuberculous endometritis and salpingitis pathogens were included in the study (commercial kit used) and processed as per the manufacturer instructions. Primers were purchased from Eurofins Genomics. Total DNA was isolated using a Magmax DNA (MagMAX DNA Multisample Ultra 2.0 Kit) from endometrial biopsies previously treated with enzymatic digestion for difficult-to-lyse bacteria. In brief, 20 mg of tissue was chopped into small pieces

and treated with Enhancer/Extraction Buffer/proteinase K at 65°C for 30 to 60 minutes under Thermomixer until the complete tissue digestion. DNA purification was performed postpretreatment process as per manufacturer's instructions.

Bacterial DNA template: All the positive controls were obtained from ATCC Culture such as *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *M. hominis* (ATCC 33131), *C. trachomatis* (VR-571B) and *N. gonorrhoeae* (ATCC 33530) *S. aureus* (ATCC 25923), *M. genitalium* (ATCC 49226), *U. urealyticum* (ATCC 276818). Clinical isolates of *S. agalactiae* NMNLC1 confirmed by Vitek2, conventional culture, and Sanger sequencing method published under NCBI accession ID OP984749.

RT-PCR assays: Applied Biosystems QuantStudio 5 PCR was used for all RT-PCR assays. The reaction mixtures contained 10 to 100 ng of DNA isolated from endometrial biopsies or commercial purified bacterial DNA as the template (positive control), 2.5 mol/L MgCl2, 0.25 mmol/L forward and reverse primers, and 5 μL of the DNA Master SYBR Green I 10× (Roche Applied Science, Mannheim, Germany), resulting in a final reaction volume of 20 μL uniplex PCR reaction contains individual target specific primer (initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 10 seconds at 95°C (denaturation), 10 seconds at 60°C (annealing), and 10 seconds at 72°C (extension) and final melt curve analysis at 95°C for 5 seconds, 15 seconds at 65°C, and temperature continuous acquisition up to 95°C; and cooling at 40°C for 30 seconds. The following quality controls were used: Each experiment used both negative and positive controls to rule out any contamination or nonspecific amplification. Positive controls comprised the specific bacterial DNA template at 50 ng concentration. Nuclease-free water was used as a template in each test as a negative control. To confirm the extraction and amplification reaction, a human target GAPDH amplification reaction was employed as an internal control (reference gene). Following amplification, a melt curve analysis was performed to differentiate the targeted PCR products from nonspecific PCR products.

Results

The analytical specificity of the DICE panel was carried out using primers against the other bacterial DNAs, insignificant cross-reactivity was detected between them. Then, the minimum detection limit of each microorganism was estimated by amplifying increasing amounts of each template DNA (0.1−100 ng DNA) alone specific to the target primers. The detection limit of these RT-PCR reactions showed a high sensitivity for the bacteria tested ranging from 0.1 to 1 ng for all microorganisms, except for *M. hominis*, which showed a minimum detection limit of 5 to 10 ng DNA. Whereas MTB below detection limit was calculated as 5 ag (attogram) equivalent to 11 copies/reaction as per manufacturer instructions. The analytical sensitivity and specificity was found to be 100% as per the study design and results obtained.

To minimize the cross-contamination and lower sensitivity, we validated and processed all samples and controls in individual reactions except for *E. coli*, and Internal control alone was used as a duplex reaction, which resulted in the same sensitivity and specificity. The melting peak profile and melting temperature were determined, defining a specific melting temperature for each amplicon, thus discriminating between specific (positive) and unspecific (negative) amplification signals (Figure A and B). In order to evaluate the efficacy of in-house designed PCR identifying the Positive polymicrobial prevalence and negative results of classical vs molecular PCR method was analyzed further.

Molecular diagnosis of CE

A total of 500 participants were enrolled in the current study, and the age range of female participants ranged from 18 to 50 years (Supplementary Table 1). The samples were stratified into three primary clinical conditions, with participant numbers distributed as follows: Abnormal bleeding (116), recurrent implantation failure or IVF failure (203), and RPL (181).

Principal findings of the study

Among the 500 samples collected from patients with confirmed endometritis through hysteroscopic and/or conventional methods, 318 (63.6%) tested positive using the molecular method, while 115 (23%) of the 500 samples showed positivity for microbial cultures targeting specific pathogens (see Table 2). The prevalent bacterial isolates included *E. faecalis* (*54 cases*, *10.8%*), *S. aureus* (*31 cases*, *6.2%*), *E. coli* (*19 cases*, *3.8%*), *S. agalactiae* (*11 cases*, *2.2%*), and other pathogens not targeted by the PCR method, such as *Streptococcus epidermis* (*1 case*, *0.2%*), *Klebsiella pneumoniae* (*2 cases*, *0.4%*), *Candida* spp. (*1 case*, *0.20%*), *and Bacillus* spp. (3 cases, 0.8%).

In contrast, the PCR method identified various pathogens, including *E. faecalis* (*95 cases*, *19%*), *E. coli* (*34 cases*, *6.8%*), *S. aureus* (*45 cases*, *9%*), *M. hominis* (*25 cases*, *5%*), *M. genitalium* (*31 cases*, *6.2%*), *S. agalactiae* (*21 cases*, *4.2%*), *U. urealyticum* (*20 cases*, *4%*), *NTM* (*26 cases*, *5.2%*), *MTB* (*6 cases*, *1.2%*), *N. gonorrhoeae* (*3 cases*, *0.6%*), *C. trachomatis* (12 cases, 2.4%), with no target pathogens amplified in 182 (36.4%) samples.

In the cohort of patients experiencing abnormal bleeding, 33 (28.4%) and 10 (8.6%) of the 116 samples tested positive using PCR and culture methods, respectively. For patients with recurrent implantation failure, 169 (64.1%) and 56 (27.6%) samples were identified as positive through PCR and culture techniques, respectively. Similarly, in the group of patients with RPL, 116 (64.1%) and 49 (27.1%) samples were confirmed positive by PCR and culture methods. In the control group, only one out of 50 samples exhibited positivity for *S. agalactiae* as determined by PCR, and no growth was observed in the culture method.

Our study findings suggest that bacterial infections are a significant factor in recurrent implantation failure

FIGURE

(A) Amplification cycles (Cp) of commercial bacterial DNA (50 ng) of the most frequent bacteria causing chronic endometritis using bacteria-specific primers. (B) Real-time polymerase chain reaction graphs show the melting peak profile and melting temperature (Tm) for each amplicon, distinguishing between specific (positive) and unspecific (negative) amplification signals

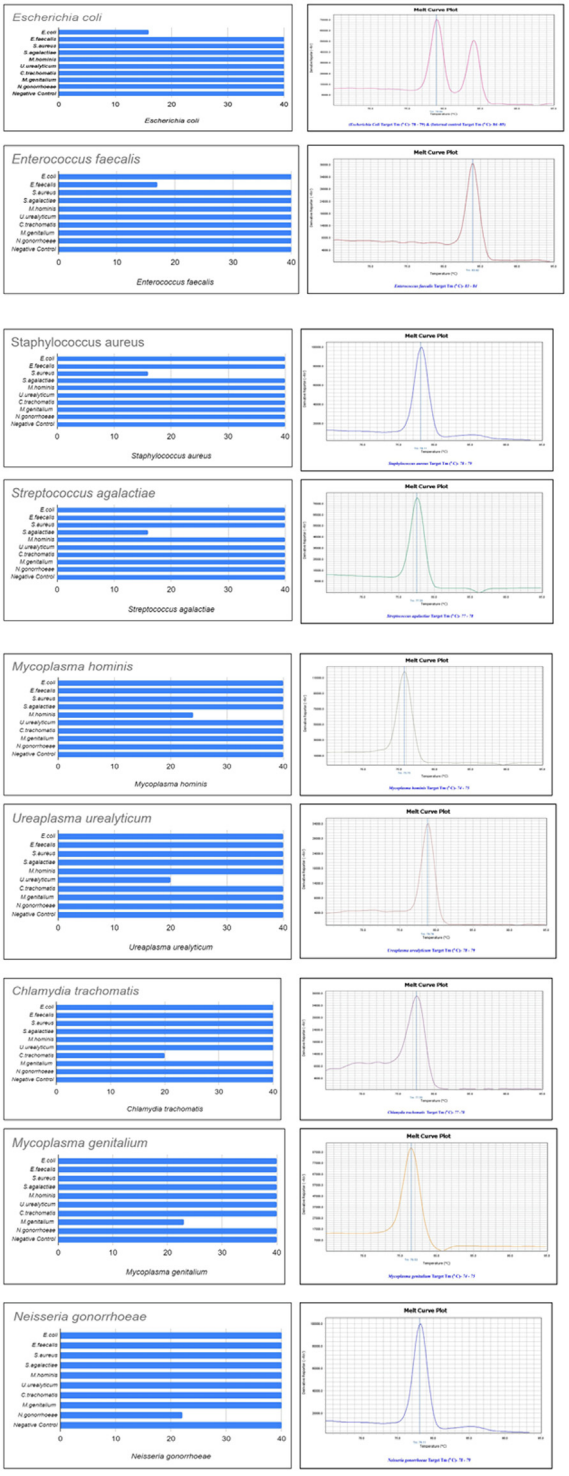

TABLE 2

Positive rate in various clinical situations utilizing molecular vs microbiological culture approaches

Clinical conditions	No of samples	*Escherichia coli*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Mycoplasma hominis*	*Ureaplasma urealyticum*	*Chlamydia trachomatis*	*Mycoplasma genitalium*	*Neisseria gonorrhoeae*	MTB	NTM	Total positives	Positive (%)
Abnormal bleeding	116 (PCR)	4 (3.45%)	5 (4.31%)	9 (7.76%)	1 (0.86%)	4 (3.45%)	3 (2.59%)	3 (2.59%)	2 (1.72%)	0	0	2 (1.72%)	33	28.4
	116 (Culture)	1 (0.86%)	2 (1.72%)	7 (6.03%)	0	-	-	-	-	-	-	-	10	8.6
Recurrent Implantation Failure	203 (PCR)	16 (7.88%)	46 (22.7%)	22 (10.8%)	12 (5.9%)	12 (5.9%)	11 (5.4%)	7 (3.4%)	19 (9.4%)	1 (0.5%)	4 (2%)	19 (9.4%)	169	83.3
	203 (Culture)	10 (4.92%)	24 (11.8%)	15 (7.4%)	7 (3.4%)	-	-	-	-	-	-	-	56	27.6
Recurrent pregnancy loss	181 (PCR)	14 (7.73%)	44 (24.3%)	14 (7.7%)	8 (4.4%)	9 (5%)	6 (3.3%)	2 (1.1%)	10 (5.5%)	2 (1.1%)	2 (1.1%)	5 (2.8%)	116	64.1
	181 (Culture)	8 (4.41%)	28 (15.5%)	9 (5.0%)	4 (2.2%)	-	-	-	-	-	-	-	49	27.1

conditions, accounting for 83.3% of cases, and a bacterial burden of 64.1% was found in cases of RPL conditions, while 28.4% were observed in cases of abnormal bleeding-related reproductive failures.

Patients with abnormal bleeding (*n*=116) exhibited the highest positive rates for *E. faecalis* (*4.31%*), *followed by E. coli* (*3.45%*), *M. hominis* (*3.45%*), *U. urealyticum* (*2.59%*), *M. genitalium* (*1.72%*), *NTM* (*1.72%*), *S. aureus* (*7.76%*), *and C. trachomatis* (*2.59%*). Patients with recurrent implantation failure (*n*=203) showed positive rates for *E. faecalis* (*22.7%*), *E. coli* (*7.88%*), *S. aureus* (*10.8%*), *S. agalactiae* (*5.9%*), *M. genitalium* (*9.4%*), *M. hominis* (*5.9%*), *U. urealyticum* (*5.4%*), *N. gonorrhea* (*0.5%*), *C. trachomatis* (*3.4%*), *MTB* (*2%*). Patients under RPL condition (*n*=181) exhibited positive rates for E. faecalis (24.3%), followed by *S. aureus* (*7.7%*), *M. hominis* (*5%*), *E. coli* (*7.73%*), *S. agalactiae* (*4.4%*), *U. urealyticum* (*3.3%*), *M. genitalium* (*5.5%*), *MTB* (*1.1%*), *and NTM* (*2.8%*).

Statistical analysis

1. Hysteroscopy (*n*=500) demonstrated a sensitivity of 100%, specificity of 50%, accuracy of 73.31%, positive predictive value (PPV) of 63.6%, negative predictive value (NPV) of 100%, false positive rate (FPR) of 50%, and false negative rate (FNR) of 0%.
2. Histology (*n*=310) exhibited a sensitivity of 97.48%, perfect specificity of 100%, high accuracy at 98.4%, PPV of 100%, NPV of 95.79%, no false positives (FPR=0%), and a low FNR of 2.52%.
3. Microbial culture (*n*=500) showed a sensitivity of 36.16%, perfect specificity of 100%, accuracy of 59.4%, PPV of 100%, NPV of 47.27%, no false positives (FPR=0%), and a high FNR of 63.84%.
4. Molecular diagnosis (*n*=500) demonstrated a sensitivity of 100%, specificity of 95.74%, accuracy of 98.42%, PPV of 97.55%, NPV of 100%, a low FPR of 4.26%, and no false negatives (FNR=0%).
5. Histology+hysteroscopy (concordant results, *n*=310) showed a sensitivity of 100%, specificity of 50%, accuracy of 72.46%, PPV of 62%, NPV of 100%, FPR of 50%, and no false negatives (FNR=0%) (Table 3).
6. Histology+hysteroscopy+microbial culture (concordant results, *n*=115) exhibited a sensitivity of 61.04%, perfect specificity of 100%, accuracy of 71.12%, PPV of 100%, NPV of 47.27%, no false positives (FPR=0%), and a moderate FNR of 38.96%.
7. Histology+molecular diagnosis (concordant results, *n*=310) demonstrated a sensitivity of 100%, specificity of 95.74%, accuracy of 98.42%, PPV of 97.55%, NPV of 100%, a low FPR of 4.26%, and no false negatives (FNR=0%).

The summary of the statistical analysis elucidated (Table 3).

Therapeutic approach

The treatment of CE primarily involves the administration of oral antibiotics tailored to the pathogen profiles identified from endometrial aspiration or biopsy. There is no universally accepted antibiotic regimen; different antibiotics and dosages have been utilized. Typically, endometrial receptivity improves following antibiotic therapy. According to the guidelines by Cicinelli et al.,[37] specific antibiotics are recommended based on the identified pathogens: ciprofloxacin 500 mg twice daily for Gram-negative and Gram-positive bacteria, amoxicillin-clavulanic acid 2 g once daily, josamycin 2 g per day for Mycoplasma or Ureaplasma, and minocycline 200 mg per day for resistant cases (Table 4).

Clinicians prescribe a regimen of Diclofenac (50 mg) combined with Trypsin Chymotrypsin (50,000 IU) for 5 days to alleviate pain and discomfort in symptomatic CE patients and provide postoutpatient endometrial sampling pain relief.[44] However, it is not mandatory for asymptomatic patients with an infectious etiology.

Comment

Despite recent investigations that have unveiled the presence of microbes, the uterine cavity was traditionally considered sterile until the advent of

TABLE 3
The comparative assessment of the molecular test vs the classic diagnostics

Test	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)
Hysteroscopy (*n*=500)	100	50	73.31	63.6	100	50	0
Histology (*n*=310)	97.48	100	98.4	100	95.79	0	2.52
Microbial culture (*n*=500)	36.16	100	59.4	100	47.27	0	63.84
Molecular Diagnosis (*n*=500)	100	95.74	98.42	97.55	100	4.26	0
Histology+hysteroscopy (concordant results, *n*=310)	100	50	72.46	62	100	50	0
Histology+hysteroscopy+microbial culture (concordant results, *n*=115)	61.04	100	71.12	100	47.27	0	38.96
Histology+molecular diagnosis (concordant results, *n*=310)	100	95.74	98.42	97.55	100	4.26	0

FNR, false negative rate; *FPR*, false positive rate; *NPV*, negative predictive value; *PPV*, positive predictive value.

TABLE 4
Antibiotic choice for pathogen-specific treatment as per CDC

S.no	Target	Antibiotics	Duration
1	Gram-negative bacteria	Ciprofloxacin 500 mg	Twice a day for 10 d
2	Gram-positive bacteria	Amoxicillin+clavulanate	Twice a day for 8 d
3	*Mycoplasma and Ureaplasma urealyticum infections*	Josamycin (macrolide)	Twice a day for 12 d
4	*Mycoplasma and U. urealyticum persistent infections*	Minocycline 100 mg	Twice a day for 12 d
5	Polymicrobial infections/anaerobic infections	Ceftriaxone 250 mg Doxycycline 100 mg orally Metronidazole 500 mg orally	IM in a single dose Twice a day for 14 d Twice a day for 14 d.
6	Tuberculous endometritis	Isoniazid, ethambutol, rifampicin, and pyrazinamide, followed by isoniazid and rifampicin	For 2 mo For another 4 mo

Source: Guidelines and Cicinelli et al. (2015).[39]

metagenomic studies.[23] Microorganisms ascend from the lower genital tract and colonize the endometrium, circumventing defense mechanisms such as the cervical mucus plug and antimicrobial peptides produced by the uterus' innate immune system. This colonization occurs in response to hormonal and proinflammatory factors, resulting in a persistent inflammatory condition of the endometrial tissue known as CE.

While CE is typically perceived as an infectious or reactive process triggered by bacterial endotoxins, it induces a type 1 immunological response (TH1) at the decidua. This response stimulates the production of pro-inflammatory cytokines and paracrine factors, ultimately leading to the attenuation of endometrial receptivity. Notably, there is limited research on endometritis in the Indian population employing culture methods, and to date, no studies utilizing molecular approaches have been published.

In our investigation, we present the prevalence rates of microorganisms in endometrial biopsy samples using both molecular and culture techniques, emphasizing the challenges associated with cultivating certain microbes (such as *C. trachomatis*, *M. hominis*, *and U. urealyticum*) due to their inherent difficulty in cultivation, as well as specimen inhibitors affecting microbial culture results. Additionally, the molecular method outperformed culture in cases of low bacterial loads that failed to yield culture positivity for Pauci-bacillary MTB and NTM pathogens, establishing its superiority in the diagnostic module.

Within the context of Indian therapeutic settings, patients often received empirical antibiotic treatment or prophylaxis, especially if they were suspected of having endometritis or before undergoing assisted reproductive procedures like IVF, IUI, and ICSI. A total of 500 samples were processed sourced from various fertility clinics across 22 states and 2 union territories in Indian Hospitals, encompassing individuals aged 18 to 51 over a period of 1 year.

Clinical implications of the study

In comparison to findings by the Haggerty & Wiesenfeld group in the USA,[24,25] our study identified *C. trachomatis* and *N. gonorrhoeae* as major pathogens, albeit at lower detection rates. Notably, our investigation, the first molecular study from India on endometrial tissue, revealed a minimal prevalence of C. trachomatis (2.4%) and *N. gonorrhoeae* (0.6%). These results align with the study by Cicinelli et al. Chlamydia and Gonorrhea are common in sexually active Indian women but are less prevalent in couples with unisexual partners due to cultural distinctions.

Endometrial TB (ETB) poses a significant health challenge in India, impacting 2% to 5% of women with infertility. The prevalence underscores the imperative consideration of ETB in infertility investigations, especially in regions burdened by high TB incidence. Contributory factors include India's elevated TB prevalence, primarily manifested as pulmonary TB, facilitating *M. tuberculosis* spread to reproductive organs. Challenges in ETB diagnosis and management encompass nonspecific symptoms and restricted access to specialized healthcare.[26] Our study identified 6 (1.2%) of 500 cases positive for MTB in endometrial tissue, underscoring the essentiality of TB diagnosis within the Indian population.

Nontuberculosis mycobacterium (NTM), a diverse group akin to *M. tuberculosis*, can cause various infections, including nontuberculous mycobacterial endometritis (NTME) within the uterine lining. Although relatively uncommon, NTME prevails in an estimated 0.5% to 1% of women with chronic pelvic pain or infertility, potentially underestimated due to diagnostic challenges. Risk factors include immunosuppression, intrauterine device use, prior endometrial procedures, and underlying gynecological conditions. In our study, 26 (5.2%) of 500 patients tested positive for NTME, notably 19 from recurrent implantation failure cases, 5 from RPL, and 2 from abnormal bleeding conditions.

The investigation conducted by the Sharma Group in India, involving 242 infertile women, reported a prevalence rate of ETB at approximately 6.2%, aligning closely with our study's findings of 4.54% among clinically

suspected CE cases.[27] Another Indian study by Yadav et al.[28] in 2020, examining 315 women with infertility, revealed a higher ETB prevalence rate of nearly 23.8%, surpassing our detection rate. Similarly, Radha Bai Prabhu et al.'s[29] study in 2019 investigated endometrial samples from infertile women with tubal illness, identifying a 6% incidence of MTB and a 23.7% prevalence of NTM. The comparatively lower rates of MTB and NTM detection in our study can be attributed to its inclusive design, encompassing patients with abnormal bleeding, recurrent implantation failure (RIF), RPL, rather than being confined to tubal defects or ETB. Notably, all identified MTB and NTM were confirmed susceptible to Rifampicin through a commercial RT-PCR kit (data not shown).

Female genital TB is treated similarly to pulmonary TB, requiring a total treatment duration of 6 months. Patients who test positive for TB are recommended to start anti-TB treatment. This treatment involves a regimen of four drugs—rifampicin (R), isoniazid (H), pyrazinamide (Z), and ethambutol (E) taken orally daily for 2 months in the intensive phase, followed by three drugs (RHE) taken orally daily for the next 4 months in the continuation phase. These guidelines are based on recommendations from the WHO and the National Tuberculosis Elimination Program of India.[40–43]

Our study demonstrated that IVF patients who responded to pathogen-specific antibiotic therapy had significantly higher clinical pregnancy rates compared to those who received empirical treatment. According to CDC guidelines, *Mycoplasma* and *Ureaplasma* infections were treated with Minocycline and Metronidazole as antimicrobial agents. Additionally, Florita LL was administered to enhance gut and vaginal microbial flora, fostering a healthy environment on the endometrial and uterine surfaces.

Notably, patients with IVF and implantation failures experienced successful outcomes following pathogen-specific antibiotic treatment, as opposed to the broad-spectrum use of doxycycline for empirical treatment. Although antibiotics are frequently used for CE treatment, the optimal regimen is still unclear. While CDC guidelines recommend several drug regimens (as shown in Table 4), such as doxycycline, metronidazole, and azithromycin.

Among individuals with noninfectious etiology, additional anti-inflammatory medications such as NSAIDs, particularly Diclofenac with chymotrypsin, have shown favorable outcomes. However, clinicians generally do not prescribe anti-inflammatory medications for patients with an infectious etiology. Further research is needed to establish an effective treatment protocol for CE.[37]

In our investigation, 180 (54.5%) of 330 clinically confirmed CE samples exhibited no amplification of target pathogens. This may be attributed to other unaddressed infections or endometritis-related variables such as altered endometrial decidualization and gene expression.[30] Changed endometrial gene expression (i.e., insulin-like growth factor 1) Different immune or immunomodulating cell compositions, such as B cells, natural killer cells, regulatory T cells (Treg), or T helper cell subpopulations Th1/Th2, Th17,[31–33] cytokine dysregulation, altered autophagy.[34]

The study suggests the inclusion of a broader spectrum of pathogens, like *Enterobacteriaceae* and Gram-positive pathogens, in CE screening. These were excluded from molecular analysis due to their absence in the culture approach, an exclusion influenced by practical considerations regarding consumable usage and its indirect impact on the study population.

A study by Cicinelli et al.[9] in 2018 revealed that the most prevalent microorganisms isolated in the endometrial cavity were common bacteria, constituting 58% of cases, consistent with our overall findings of 63.6%. Comparisons between the prevalence rates of endometrial cultures in Cicinelli et al.[35] study vs our study indicate slight variations: *S. aureus* (6.2% vs 4.54%), *E. faecalis* (10.8% vs 8.18%), *E. coli* (3.8% vs 3.94%), Yeast (1% vs 0.05%), and *S. agalactiae* (2.2% vs 3.3%). These variations, contrary to other studies, are corroborated by our molecular technique results. *E. faecalis* emerges as the most commonly identified pathogen across all trials, consistent with our study's outcomes.

A standardized definition or diagnostic method for CE is currently lacking, with most fertility practitioners relying on histological examination of endometrial samples to identify plasma cells in the uterine stroma. However, this approach's accuracy can be compromised by various factors, such as inflammatory cell infiltration, stromal cell proliferation, plasmacytoid stromal cell appearance, or a pronounced predecidual reaction in the late secretory endometrium. Moreover, inter- and intraobserver variability exists in this analysis. Syndecan-1 (CD138) immunohistochemistry staining, with its heightened sensitivity in recognizing plasma cells compared to traditional methods, is considered crucial for precise diagnosis, emphasizing the importance of an experienced pathologist. Diverse approaches are employed across studies to quantify plasma cells and define CE, contributing to variations in reported prevalence and raising concerns about the exclusive reliance on histology for therapeutic decision-making.[36]

Strength and limitations

Our findings indicate that PCR-based detection, by overcoming the limitations of individual traditional methods, yields results comparable to a combined approach. The molecular method boasts several advantages: it is highly sensitive, capable of detecting small amounts of bacterial DNA irrespective of cultivability, facilitates severity estimation of CE, enables testing on frozen or fixed samples, and provides rapid results compared to bacterial culture. However, drawbacks include the inability to confirm viable bacteria and the need to determine the minimum amount of bacterial DNA causing the disease, as its presence in some women may be inconsequential depending on the host response and does not distinguish between acute and CE.

Conclusion

In summary, our research showcases the remarkable efficacy of (DICE panel) RT-PCR in swiftly and precisely identifying the distinctive bacterial DNA associated with CE in endometrial samples. The molecular approach excels in conjunction with conventional diagnostic methods like hysteroscopy, histology, and microbial culture, providing a practical, rapid, and cost-effective solution for diagnosing CE. This study unequivocally highlights the effectiveness of the molecular method, showcasing its precision and sensitivity in detecting pathogens linked to CE. Moreover, it seamlessly aligns with findings from histology and hysteroscopy, further solidifying its value in clinical practice. ■

CRediT authorship contribution statement

Murugan Nandagopal: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chirayu Padhiar:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition. **Mayur Abhaya:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Uma Bansal:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Prakash Ghambir:** Writing – review & editing, Validation, Supervision, Formal analysis.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xagr.2024.100377.

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