GYNECOLOGY

The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology

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BACKGROUND: Chronic endometritis is a persistent inflammation of the endometrial mucosa caused by bacterial pathogens such as Enterobacteriaceae, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Mycoplasma*, and *Ureaplasma*. Although chronic endometritis can be asymptomatic, it is found in up to 40% of infertile patients and is responsible for repeated implantation failure and recurrent miscarriage. Diagnosis of chronic endometritis is based on hysteroscopy of the uterine cavity, endometrial biopsy with plasma cells being identified histologically, while specific treatment is determined based on microbial culture. However, not all microorganisms implicated are easily or readily culturable needing a turnaround time of up to 1 week.

OBJECTIVE: We sought to develop a molecular diagnostic tool for chronic endometritis based on real-time polymerase chain reaction equivalent to using the 3 classic methods together, overcoming the bias of using any of them alone.

STUDY DESIGN: Endometrial samples from patients assessed for chronic endometritis (n = 113) using at least 1 or several conventional diagnostic methods namely histology, hysteroscopy, and/or microbial culture, were blindly evaluated by real-time polymerase chain reaction for the presence of 9 chronic endometritis pathogens: *Chlamydia trachomatis*, *Enterococcus*, *Escherichia coli*, *Gardnerella vaginalis*, *Klebsiella pneumoniae*, *Mycoplasma hominis*, *Neisseria gonorrhoeae*, *Staphylococcus*, and *Streptococcus*. The sensitivity and specificity of the molecular analysis vs the classic diagnostic techniques were compared in the 65 patients assessed by all 3 recognized classic methods.

RESULTS: The molecular method showed concordant results with histological diagnosis in 30 samples (14 double positive and 16 double negative) with a matching accuracy of 46.15%. Concordance of molecular and hysteroscopic diagnosis was observed in 38 samples (37 double positive and 1 double negative), with an accuracy of 58.46%. When the molecular method was compared to microbial culture, concordance was present in 37 samples (22 double positive and 15 double negative), a matching rate of 56.92%. When cases of potential contamination and/or noncultivable bacteria were considered, the accuracy increased to 66.15%. Of these 65 patients, only 27 patients had consistent histological + hysteroscopic diagnosis, revealing 58.64% of nonconcordant results. Only 13 of 65 patients (20%) had consistent histology + hysteroscopy + microbial culture results. In these cases, the molecular microbiology matched in 10 cases showing a diagnostic accuracy of 76.92%. Interestingly, the molecular microbiology confirmed over half of the isolated pathogens and provided additional detection of nonculturable microorganisms. These results were confirmed by the microbiome assessed by next-generation sequencing. In the endometrial samples with concordant histology + hysteroscopy + microbial culture results, the molecular microbiology diagnosis demonstrates 75% sensitivity, 100% specificity, 100% positive and 25% negative predictive values, and 0% false-positive and 25% false-negative rates.

CONCLUSION: The molecular microbiology method describe herein is a fast and inexpensive diagnostic tool that allows for the identification of culturable and nonculturable endometrial pathogens associated with chronic endometritis. The results obtained were similar to all 3 classic diagnostic methods together with a degree of concordance of 76.92% providing an opportunity to improve the clinical management of infertile patients with a risk of experiencing this ghost endometrial pathology.

Key words: bacterial pathogens, chronic endometritis, endometrial microbiome, histology, hysteroscopy, microbial culture, molecular microbiology diagnosis, next-generation sequencing, real-time polymerase chain reaction

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Introduction

The Human Microbiome Project has highlighted the importance of microorganisms and their genomes in human health and disease,[1] and has brought to light the value of detecting dysbiotic microbiomes to facilitate the improvement of clinical management. Chronic endometritis is a persistent inflammation of the endometrial mucosa caused by the presence of bacterial pathogens in the uterine cavity. The most common infectious agents responsible for chronic endometritis are *Enterococcus faecalis*, *Enterobacteriaceae*, *Streptococcus* species, *Staphylococcus* species, *Gardnerella vaginalis*, and *Mycoplasma* species as well as genital pathogens associated with sexually transmitted infections, such as *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*.[2,3]

AJOG at a Glance

Why was this study conducted?

Chronic endometritis diagnosis still depends on the method used. The aim of this study is to develop a new molecular method for the diagnosis of chronic endometritis, overcoming the bias of the current methods.

Key findings

Molecular microbiology can be used to improve diagnosis and management of chronic endometritis in asymptomatic infertile patients.

What does this add to what is known?

Molecular microbiology can detect bacterial pathogens causing chronic endometritis and could be useful to guide a target therapy for this ghost endometrial condition.

Chronic endometritis is often clinically silent and rarely suspected and diagnosed, although it can be accompanied by symptoms like pelvic pain, dysfunctional uterine bleeding, dyspareunia, and leukorrhea.[4] The actual prevalence in the general population is ill-defined, although it has been estimated to be between 0.8-19%.[5] Even if clinically silent, chronic endometritis has been suggested to diminish the success rates of both spontaneous and assisted reproductive technology conceptions as well as contributing to obstetric and neonatal complications.[6-12] The prevalence of chronic endometritis in infertile patients has been estimated at 2.8-39%,[9,13-19] but can be as high as 60% or 66% in women diagnosed with unexplained recurrent miscarriage or repeated implantation failure, respectively.[20,21]

The diagnosis of chronic endometritis is difficult because there are no typical clinical or ultrasound findings. Classic diagnostic techniques of chronic endometritis rely on histology, which is based on the identification of plasma cells in the endometrial stroma,[4] but this method is nonspecific and dependent on the date of the menstrual cycle when sampling occurs. Considering these limitations, hysteroscopy and microbial culture are also often used for chronic endometritis diagnosis.[3,15] Hysteroscopic diagnosis of chronic endometritis relies on subjective characteristics identified by the reproductive endoscopist such as stromal edema, focal or diffuse epithelial hyperemia, and/or the presence of micropolyps. The identification of endometrial pathogens by microbial culture is the only method that provides objective information for targeted therapy. Its use has resulted in an improvement of reproductive outcome in women with recurrent miscarriage and repeat implantation failure;[20,21] however, endometrial bacterial culture is not routinely performed because it has a long turnaround time, and not all microorganisms responsible for chronic endometritis are culturable.

To improve and personalize the state of the art for diagnosing and treating chronic endometritis, researchers must determine the identity and pathogenicity of the microbes prone to produce an endometrial infection. Molecular methods have revolutionized the detection and characterization of microorganisms in a broad range of medical fields including virology, mycology, parasitology, microbiology, and dentistry.[22] For instance, in public health, the screening of *Mycobacterium tuberculosis* by polymerase chain reaction (PCR) allows for early recognition and optimized treatment.[23] Along with conventional PCR techniques, real-time (RT)-PCR has an ever-increasing role in clinical diagnostics based on its capacity to detect difficult-to-culture bacteria and generate both qualitative and quantitative results in an accurate and rapid manner.[24] The aim of this study is to compare, in the same infertile patients, the diagnostic accuracy of the molecular microbiology tool with the traditional chronic endometritis diagnostic methods, ie, endometrial histology, hysteroscopy, and/or microbial culture, by assessing the presence of 9 specific chronic endometritis pathogens by RT-PCR and next-generation sequencing (NGS).

Materials and Methods

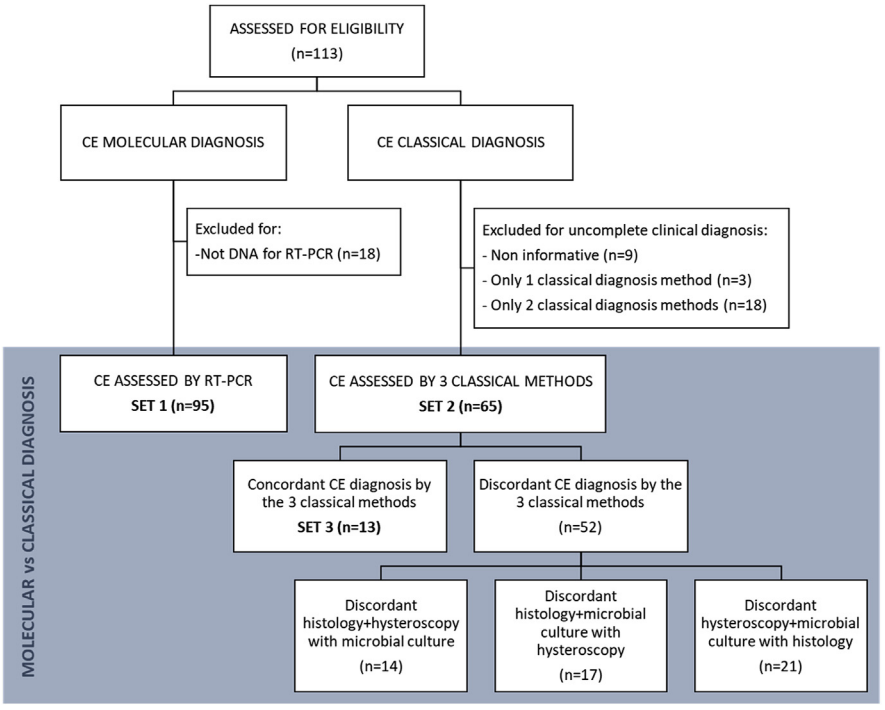
Study design

Endometrial samples from 113 patients subjected to chronic endometritis diagnosis using endometrial histology, hysteroscopy, and/or microbial culture were blindly evaluated for the presence of 9 chronic endometritis pathogens by RT-PCR using paired endometrial samples. Then, sensitivity and specificity of the molecular analysis and the classic diagnostic techniques were compared in 65 patients with chronic endometritis results assessed by all 3 recognized classic methods (Figure 1). In parallel, endometrial samples of negative controls based on histology and microbial culture ($n = 10$) were evaluated for the presence of chronic endometritis pathogens by molecular microbiology.

Study participants

Participants involved in this study were 21- to 53-year-old infertile patients recruited (E.C.) at the Second Unit of Obstetrics and Gynecology, Department of Biomedical and Human Oncological Science, University of Bari, Bari, Italy, undergoing in vitro fertilization treatment. Patients were diagnosed and treated for chronic endometritis using either a single or various classic diagnostic techniques. Frozen endometrial biopsy specimens were sent blindly to Igenomix SL for molecular microbiology diagnosis by RT-PCR. Also, endometrial tissue from women with negative results for chronic endometritis was analyzed using RT-PCR. This group of negative controls consisted of women undergoing surgery for benign ovarian conditions (oophorectomy for dermoid cyst with exclusion of endometriosis) or operated on due to myomas (myomectomy) ($n = 6$), and women treated with antibiotics for previous chronic endometritis with no current signs of the disease at the time of sample collection by either histology and microbial culture ($n = 4$). The ethical committee of the Second Unit of

FIGURE 1
Diagram of study and distribution of population investigated



Molecular diagnosis of chronic endometritis (CE) performed by real-time (RT) polymerase chain reaction (PCR). Classic diagnosis of CE includes histology, hysteroscopy, and microbial culture.

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Obstetrics and Gynecology, Department of Biomedical and Human Oncological Science, University of Bari, Bari, Italy, approved the study (register number 4880). All women signed an informed consent form agreeing to the comparative analysis of the techniques performed for the routine diagnosis of chronic endometritis vs the molecular diagnosis.

Histological diagnosis

Patients underwent endometrial sampling using a 3-mm Novak curette connected to a 20-mL syringe. To minimize the risk that endometrial cultures might be contaminated by the vaginal flora, after placing a vaginal speculum, the Novak cannula was inserted under visual control into the uterine cavity avoiding any contact with the vaginal walls. Histological examination was performed blindly by 2 pathologists who were unaware of the results of the other diagnostic techniques. Paraffin-embedded endometrial specimens were used for the study. Samples were fixed overnight in 4% formaldehyde (in phosphate buffer, pH 7.3, Nacalai Tesque, Kyoto, Japan) and embedded in paraffin (Nacalai Tesque). Microsections of 5 μm were stained with hematoxylin-eosin. The inflammatory status of the endometrium was graded based on the presence of stromal infiltrate dominated by lymphocytes and plasma cells, and a spindle cell change of stromal cells.[25] Infiltration showing aggressive behavior against glands, inflammatory cell-gathering inside glands, or a more structured infiltration was also relevant. Micropolypoid vegetations formed in the endometrium are macroscopic evidence of an inflammation-related process and can be used as markers for this pathology. All cases diagnosed with chronic endometritis by histological criteria were confirmed by antisyndecan-1 (CD138) immunohistochemistry as previously described.[25]

Hysteroscopy

All hysteroscopies were performed by 2 physicians using a lens-based 2.7-mm outer diameter mini-telescope with a 105-degree angle of visual field equipped with a 4.5-mm outer diameter double-flow operative sheath (Karl Storz, Tuttlingen, Germany). Hysteroscopies were performed in the follicular phase (cycle day 7-12). Saline was employed to distend the uterine cavity at a pressure generated by a simple drip from a bag suspended 1 m above the patient. A 300-W light source with a xenon bulb and a high-definition digital camera (Karl Storz) were used. During hysteroscopy, both the anterior and posterior uterine walls were thoroughly examined by passing the hysteroscope parallel to the endometrial surface to identify any surface irregularity.

The following criteria were used for the hysteroscopic diagnosis of chronic endometritis: the presence of stromal edema, focal or diffuse periglandular hyperemia, and micropolyps of <1 mm in size.[21-26] The hysteroscopic images were reviewed in the patient database at the Second Unit of Obstetrics and Gynecology, Department of Biomedical and Human Oncological Science, University of Bari, Bari, Italy.

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 chronic endometritis pathogens. In particular, to *detect C. trachomatis, N. gonorrhoeae, U. urealyticum*, *U. parvum*, and *Mycoplasma hominis*, all of which are noncultivable strains recoverable from the genital tract, a multiplex RT method (AnyplexTM II STI-7 Detection [V1.1]; Seegene, Seoul, Republic of Korea) was used according to the manufacturer's instructions. To detect the presence of culturable microorganisms (*E. faecalis*, *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, and yeasts), the endometrial samples were inoculated onto culture media either directly or following enrichment in brain heart infusion medium. These media consisted of columbia-colistin-nalidix acid agar with 5% sheep

blood, which is a selective and differential medium commonly used in the isolation of gram-positive organisms from mixed clinical specimens, and MacConkey agar and mannitol salt agar for the isolation of gram-negative bacteria and *Staphylococcus aureus*. Consequently, the growth of the bacteria indicated above was considered positive when >10,000 colony-forming units were isolated from the culture. In the case of a positive culture, antibiotic sensitivity and resistance were tested by Vitek 2 (BioMérieux). According to microbiology guidelines, the presence of *Staphylococcus epidermidis* was considered to indicate contamination.

Molecular diagnosis

Frozen endometrial specimens were sent to Igenomix SL for the blind identification of chronic endometritis pathogens by RT-PCR using specific primers for the 9 most common bacteria responsible for causing chronic endometritis (*C. trachomatis*, *Enterococcus* species, *E. coli*, *G. vaginalis*, *Klebsiella pneumoniae*, *M. hominis*, *N. gonorrhoeae*, *Staphylococcus* species, and *Streptococcus* species). A literature search was conducted to identify species- and genus-specific primers for the bacteria of interest, plus 1 primer for the family *Enterobacteriaceae* including *E. coli* and *K. pneumoniae* (Table 1). The primers used were reevaluated and subjected to Basic Local Alignment Search Tool searches using the National Center for Biotechnology Information genomic database. All primers were purchased from Integrated DNA Technologies (Skokie, IL).

Total DNA was isolated using a QIAamp cador pathogen mini kit (Qiagen, Hilden, Germany) from endometrial biopsies previously treated with enzymatic digestion for difficult-to-lyse bacteria. For this digestion, 25 mg of tissue were cut into small pieces and treated with proteinase K at 56°C for 3 hours under agitation or until the tissue was completely lysed. Then, the sample was mixed with ATL buffer (Qiagen) and disrupted mechanically in a TissueLyser LT (Qiagen) for 5 minutes at 50 Hz, using stainless-steel beads. After these pretreatments, bacterial nucleic acids were purified following the manufacturer's instructions.

Bacterial DNA template

A panel of DNA templates of bacteria causing chronic endometritis was selected to evaluate the specificity of the RT-PCR assays. A total of 11 DNA templates were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), including templates of common microorganisms of the disease, *Enterococcus faecalis* (DSMZ 2570), *Escherichia coli* (DSMZ 1116), *G vaginalis* (DSMZ 4944), *K pneumoniae* (DSMZ 30104), *Mycoplasma hominis* (DSMZ 25592), *Staphylococcus epidermidis* (DSMZ 1798), *Staphylococcus haemolyticus* (DSMZ 20263), *Streptococcus agalactiae* (DSMZ 2134), and *Streptococcus gallolyticus* (DSMZ 20065), as well as templates of sexually transmitted disease pathogens, *Chlamydia trachomatis* (DSMZ 19411) and *N gonorrhoeae* (DSMZ 15130).

RT-PCR assays

All RT-PCR reactions were performed on a LightCycler 480 II (Roche Diagnostics, Almere, The Netherlands). Reaction mixtures contained 200 ng of DNA isolated from endometrial biopsies or commercial purified bacterial DNA as the template, 2.5 mmol/L $MgCl_2$, 0.25 μmol/L forward and reverse primers, and 1 μL of the LightCycler FastStar DNA Master SYBR Green I 10x (Roche Applied Science, Mannheim, Germany) resulting in a final reaction volume of 20 μL. The cycling program was as follows: an initial denaturation at 95°C for 10 minutes; amplification for 45 cycles of 10 seconds at 95°C, 10 seconds at 57°C, and 50 seconds at 72°C; melting curve analysis for 5 seconds at 95°C, 15 seconds at 65°C and a temperature continuous acquisition up to 95°C (ramp rate of 0.11°C/s); and cooling for 30 seconds at 40°C.

Each experiment included negative and positive controls to determine any possible contamination and unspecific amplification. Positive controls included a mix of all the bacterial DNA templates at a concentration of 10,000 genomes each. Negative controls included all DNA templates except the microorganism to be evaluated for each assay. The genome size of each bacterium was used for the calculation of genomes/ng of DNA.[27]

A melting curve analysis was made after amplification to distinguish the targeted PCR products from nonspecific PCR products. The concentration of microorganism in each sample was calculated comparing the crossing point-PCR-cycle (Cp) values obtained from the sample with the Cp values of the positive control.

16S ribosomal RNA sequencing

Endometrial microbiome profiles were obtained by NGS of the bacterial gene encoding for the 16S ribosomal RNA (rRNA) using the Ion 16S metagenomics kit (ThermoFisher Scientific, Waltham, MA), which includes 2 primer sets (V2-4-8 and V3-6, 7-9) that selectively amplify the hypervariable regions (V2-4-8 and V3-6, 7-9) of the 16S ribosomal subunit. The amplified fragments were sequenced on the Ion S5 XL system (ThermoFisher Scientific). The results were analyzed using the Metagenomics application of Ion Reporter software 5.0 (ThermoFisher Scientific) using default parameters as previously described.[28] Briefly, the QIIME package (http://qiime.org/) and Greengenes database, Version 13_8 (http://qiime.org/home_static/dataFiles.html), were used for taxonomic assignment. QIIME was used to calculate alpha diversity and rarefaction curves before filtering. Shannon methods were employed to analyze the biodiversity within a group of samples. Positive controls of *E. coli* DNA and negative controls were included to detect any contamination from reagents.

Statistical analysis

The comparative assessment of the molecular test vs the classic diagnostics methods considered (individually or combined) to be the gold standard were calculated as follows[29]:

- Sensitivity: percentage of true-positive cases among the total positives in the gold standard test.

TABLE 1
List of primers used for polymerase chain reaction amplification of selected microorganisms

Bacteria	Target gene	Primers	Amplicon length, bp	Reference
Chlamydia trachomatis	16S rRNA	F: GGATCCGTAAGTTAGACGAAATTTTG R: TTTAATGCGAAAGGAAATCTGATTG	83	41
Enterobacteriaceae	*rpoB*	F: CAGGTCGTCACGGTAACAAG R: GTGGTTCAGTTTCAGCATGTAC	512	42
Enterococcus species	*rpoB*	F: AGAGAGTAAGGTCCGATTGAAC R: GGTTGTTTCCCGTATTATGC	370	42
Escherichia coli	16S rRNA	F: AGAAGCTTGCTCTTTGCTGA R: CTTTGGTCTTGCGACGTTAT	120	43
Gardnerella vaginalis	16S rRNA	F: TTACTGGTGTATCACTGTAAGG R: CCGTCACAGGCTGAACAGT	320	27
Klebsiella pneumoniae	*gltA*	F: ACGGCCGAATATGACGAATTC R: AGAGTGATCTGCTCATGAA	68	44
Mycoplasma hominis	16S rRNA	F: CATGCATGTCGAGCGAGGTT R: CCATGCGGTTCCATGCGT	129	45
Neisseria gonorrhoeae	16S rRNA	F: GTTTCAGCGGCAGCATTCA R: CCGGAACTGGTTTCATCTGATT	102	46
Staphylococcus species	*rpoB*	F: CAGGAGAAGTTAAAGAACAAGAAG R: GTGAACGAACTAATTGAGATACG	118	42
Streptococcus species	*tuf*	F: GTACAGTTGCTTCAGGACGTATC R: ACGTTCGATTTCATCACGTTG	197	47

rRNA, ribosomal RNA.
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- Specificity: percentage of true-negative cases among the total negatives in the gold standard test.
- Accuracy: percentage of correct assessments on the total assessments.
- Positive predictive value: percentage of true-positive cases among the total positives in the molecular test.
- Negative predictive value: percentage of true-negative cases among the total negatives in the molecular test.
- False-positive rate: percentage of false-positive cases among the total negatives in the gold standard test.
- False-negative rate: percentage of negative cases among the total positives in the gold standard test.

Results

Analytical specificity of RT-PCR assays

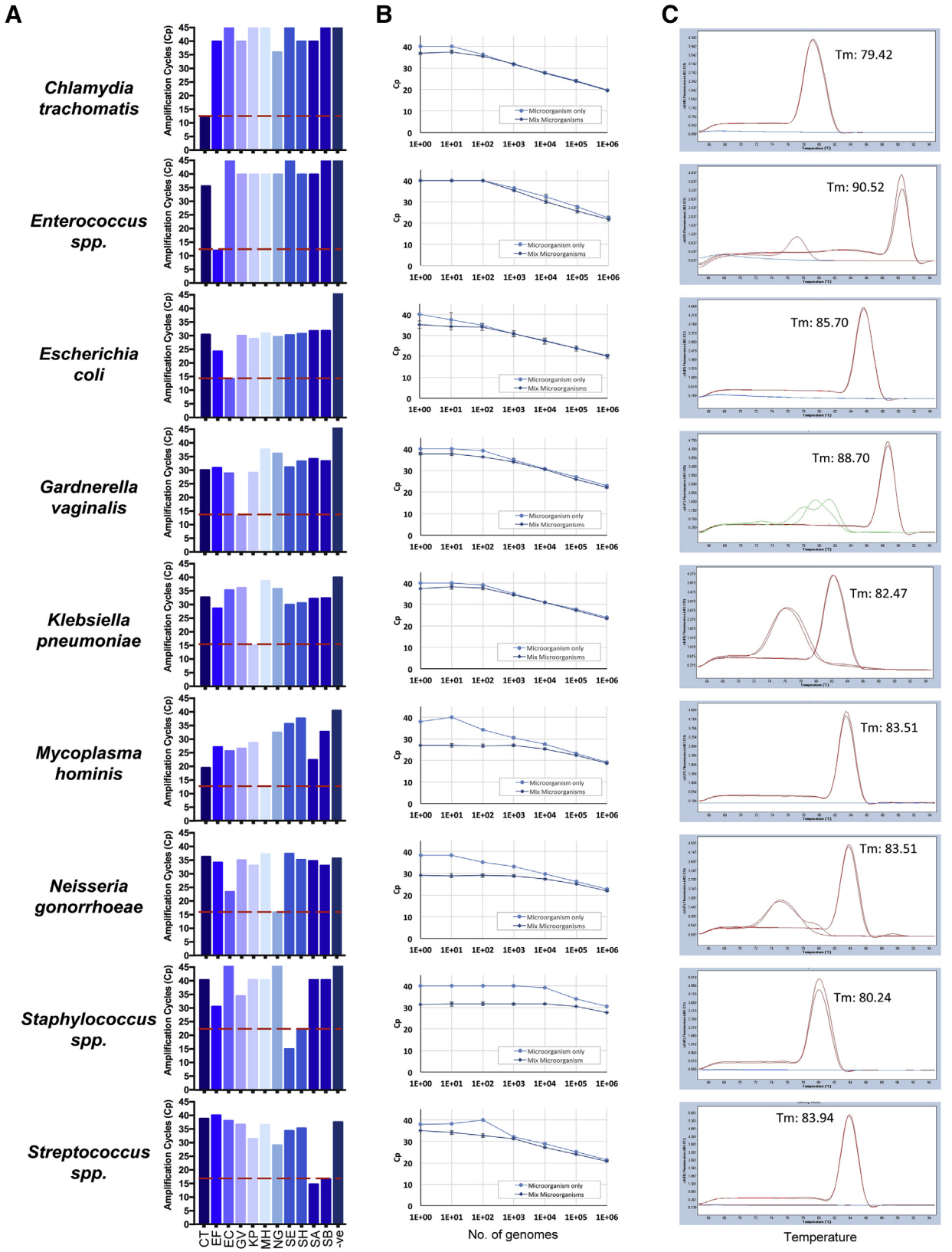
The analytical specificity of the molecular microbiology assay was verified using specific primers for the most common bacteria causing chronic endometritis using 30 ng of commercial bacterial DNA isolated from chronic endometritis bacteria as a template. All primer sets resulted in specific amplification of their own bacterial DNA templates, with Cp values ranging from 12-20 compared to the negative control of ultrapure water with the lowest Cp value at 35 for *N. gonorrhoeae*, while the majority of them did not amplify after 40 amplification cycles. When each primer pair was tested against the other bacterial DNAs, insignificant cross-reactivity was detected between them, with a minimum difference of 10 amplification cycles between the specific and nonspecific amplifications (Figure 2, A). Then, the minimum detection limit of each microorganism in the RT-PCR was assessed by amplifying increasing amounts of each template DNA (0-1,000,000 genomes) alone or in a complex mixture of bacterial DNA consisting of 1,000,000 genomes from the other microorganisms included in the molecular method. The detection limits of these RT-PCR reactions showed the high sensitivity of the molecular method for the different bacteria tested ranging from 10-1000 genomes for all microorganisms, except for staphylococci, which showed a minimum detection limit of 10,000 genomes, equivalent to the microbial culture technique in which >10,000 colony-forming unit is considered a positive test (Figure 2, B).

To minimize possible bias introduced by the cross-reactions in subsequent experiments, the positive control was defined as a mix of all the bacterial DNA templates at a concentration of 10,000 genomes each, while the negative control was a mix of all DNA templates except for the bacterial DNA to be detected. Then, for each independent assay, the melting peak profile and melting temperature were determined, defining a specific melting temperature value for each amplicon, thus discriminating between specific (positive) and unspecific (negative) amplification signals (Figure 2, C).

Molecular diagnosis of chronic endometritis using RT-PCR

DNA was isolated from 113 endometrial specimens of women being assessed for chronic endometritis using any of the

FIGURE 2
Analytical specificity of molecular microbiology



classic methods. From these, 18 samples did not yield enough standard-quality DNA for analysis. The remaining 95 (set 1) were assessed by RT-PCR with the primers indicated in Table 1 for the microorganisms *Enterobacteriaceae*, *Enterococcus* species, *E. coli*, *G. vaginalis*, *K. pneumoniae*, *M. hominis*, *Staphylococcus* species, and *Streptococcus* species. Additionally, other pathogens causing sexually transmitted diseases, such as *C. trachomatis* and *N. gonorrhoeae*, were included in the analysis. The amplified signal for each microorganism was considered positive when the threshold cross-point value (Cp) obtained was equal or lower than the Cp value of the positive control and the melting temperature matched with that established in the positive control.

Using this molecular analysis, 42 of the 95 endometrial samples were negative for chronic endometritis (44.21%), while 53 were positive (55.79%) for ≥1 of the pathogens tested (58.5% and 41.5%, respectively) (Figure 3, A). The pathogens most commonly represented in the samples analyzed by RT-PCR were streptococci, while *C. trachomatis* and *N. gonorrhoeae* were undetectable in all tested samples (Figure 3, B).

Comparison of chronic endometritis diagnosis based on molecular microbiology vs classic methods

The results of the molecular microbiology of chronic endometritis were compared with the classic diagnostic methods: histology, hysteroscopy, and microbial culture (Figure 1). For this comparison, 30 patients were excluded for lacking results from ≥1 of the 3 classic methods, so the comparison was finally performed for 65 patients (set 2) with a chronic endometritis diagnosis derived from classic techniques (Table 2).

Using molecular analysis, 27 of the 65 endometrial samples were negative for chronic endometritis (41.54%) while 38 were positive (58.46%) for ≥1 of the pathogens tested (32.31% and 26.15%, respectively) (Figure 3, A). The most commonly represented bacteria in the endometria analyzed by RT-PCR were streptococci, which accounted for 45.78% of the total pathogens detected (Figure 3, C).

Molecular microbiology vs histology

Histological diagnosis alone was positive in 25 of 65 patients investigated (38.46%) while the molecular microbiology was positive in 38 cases (58.46%). Concordant results were observed in 30 samples (14 double positives and 16 double negatives) giving a matching accuracy of 46.15%. Interestingly, in 24 (68.57%) of 35 samples with contradictory results, bacterial DNA was clearly identified by RT-PCR while the histological analysis was negative (Table 2).

Molecular microbiology vs hysteroscopy

Hysteroscopic-based diagnosis was positive in 63 of 65 patients investigated (96.92%), while the molecular microbiology was positive in 38 cases. Concordance was observed in 38 samples (37 double positives and 1 double negative) showing a matching accuracy of 58.46%. In 26 (96.29%) of 27 discordant patients, bacterial DNA was not identified in the paired endometrial sample obtained after positive hysteroscopic diagnosis of chronic endometritis. Interestingly, only 1 sample with no signs of chronic endometritis in the hysteroscopy was positive for *Streptococcus* species (Table 2).

Molecular microbiology vs microbial culture

Microbiological culture was positive in 34 of 65 patients tested for chronic endometritis by either histology or hysteroscopy (52.30%) while molecular diagnosis was positive in 38 of these cases (58.46%). Concordance between molecular microbiology and microbial culture was present in 37 patients (22 double positive and 15 double negative) showing a matching rate of 56.92%. However, when microbiological results were analyzed in detail, we found several cases in which the culture was either contaminated by *S. epidermidis* (case 28), or presented microorganisms that were not tested in the molecular diagnosis: *Ureaplasma* species and *Candida albicans* (specifically cases 20 and 48). Also, the molecular microbiology allowed for the identification of *G. vaginalis*, which is seldom cultured, causes chronic endometritis, and was not identified by classic microbial culture in some samples (cases 2 and 5). Taking these cases into account, the comparison of these 2 methods demonstrated an accuracy of 66.15% (Table 2). From these 22 double-positive cases, 11 RT-PCR cases were confirmed by isolation of the same pathogens. Moreover, in 4 cases, *G. vaginalis* DNA was identified, providing additional information over the classic microbial culture.

Molecular microbiology vs histology + hysteroscopy

Because histology and hysteroscopy are subjective methods with the highest discordant results, we compared the molecular diagnostic method to both. From the 65 patients analyzed by all methods (histology, hysteroscopy, and microbial culture), only 27 presented with consistent histology + hysteroscopy results (41.54% concordance rate), with

A, Represents amplification cycles (Cp) of commercial bacterial DNA (30 ng) of most common bacteria causing chronic endometritis using specific primers for these bacteria. **B**, Detection limit of each bacterial DNA in molecular method. Each set of primers was tested against increasing amounts of bacterial DNA (0; 1; 10; 100; 1000; 10,000; 100,000, and 1,000,000 genomes) alone or in complex mix of microorganisms containing 1,000,000 genomes of each remaining bacterial species included in molecular method. **C**, Graphs obtained from real-time polymerase chain reaction show melting peak profile and melting temperature (Tm) for each amplicon, discriminating specific (positive) and unspecific (negative) amplification signals.

CT, *Chlamydia trachomatis*; EC, *Escherichia coli*; EF, *Enterococcus faecalis*; GV, *Gardnerella vaginalis*; KP, *Klebsiella pneumoniae*; MH, *Mycoplasma hominis*; NG, *Neisseria gonorrhoeae*; SA, *Streptococcus agalactiae*; SB, *Streptococcus bovis*; SE, *Staphylococcus epidermidis*; SH, *Staphylococcus hominis*; -ve, negative control.

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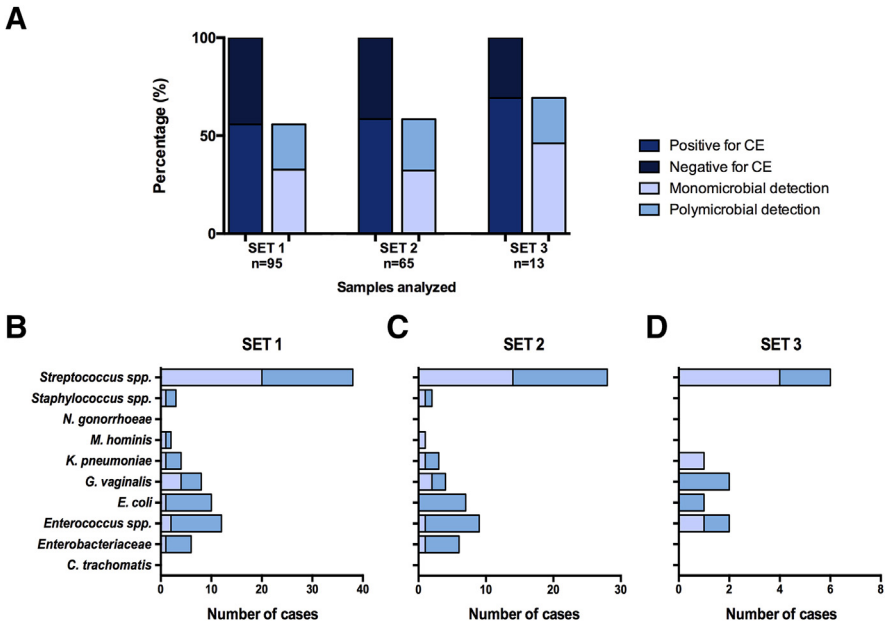
FIGURE 3
Molecular diagnosis of chronic endometritis (CE)



A, Proportion of positive and negative cases of CE using molecular method and percentage of positive cases with detection of ≥1 microorganisms in samples from set 1 (95 samples analyzed by real-time polymerase chain reaction), set 2 (65 samples used for comparison with classic diagnosis), and set 3 (13 samples with concordant results by 3 classic methods). Number of cases in which each targeted pathogen is detected alone or in combination with other bacteria in **B**, set 1; **C**, set 2; and **D**, set 3.

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25 double-positive and 2 double-negative results (Table 2). In this group, 13 cases were also consistent with microbial culture, while 14 differed from microbial culture (Figure 1). From those 27 patients, RT-PCR showed concordant results in 15 cases (14 positives and only 1 negative) with an accuracy of 55.55%. Interestingly, 38 of 65 patients (58.46%) presented with opposite results between histology and hysteroscopy, and in all negative cases for histology were positive based on hysteroscopy. In these cases, the detection of bacterial DNA by RT-PCR (23 of 38 cases) was always coincident with positive hysteroscopy, while negative diagnosis of chronic endometritis using the molecular method (15 of 38 samples) always matched with the negative histological diagnosis.

Molecular microbiology vs histology + hysteroscopy + microbial culture

From the 65 samples with chronic endometritis results from all 3 classic methods, only 13 (20%) had concordant results from all 3 (Figure 1). The molecular analysis of this set of samples showed that 4 of the 13 endometrial samples were negative for chronic endometritis (30.8%), while 9 were positive (69.2%) for ≥1 of the pathogens tested (66.7% and 33.3%, respectively) (Figure 3, A). The most detected pathogen in these samples was *Streptococcus* species, followed by *Enterococcus* species and *G. vaginalis* (Figure 3, D).

The only patient diagnosed as negative based on the 3 classic methods was also negative for the molecular evaluation of chronic endometritis (Figure 4, A). From the remaining 12 that were positive for chronic endometritis based on all 3 classic methods, 9 were also positive based on RT-PCR, while 3 showed discordant results. In total, 10 of 13 cases presented with similar results with an accuracy of 76.92% (Table 2). In 5 of the 9 cases with positive results for microbial culture and RT-PCR, the microorganisms detected were the same, and in 2 of them, *G. vaginalis* was also detected by RT-PCR together with other pathogens (cases 8 and 15), providing additional information to the microbial culture (Figure 4, A). To confirm these results, endometrial biopsies from these 13 diagnostic concordant patients were subjected to bacterial 16S rRNA sequencing to assess their full endometrial microbiome. The results of NGS for the bacterial 16S rRNA gene confirmed the detection of bacterial DNA in 12 of 13 endometrial samples, since 1 sample did not yield sequencing results (case 24). The number of mapped reads per sample was between 27,178 and maximum of 231,538, with an average value of 117,369. The data of mapped sequences and Shannon index for each sample are detailed in Table 3. The overall analysis of the endometrial microbiome in those 12 samples showed that the most represented genus was *Lactobacillus*, followed by 2 bacterial pathogens associated with chronic endometritis: *Streptococcus* and *Gardnerella*. Also, in the 20 most abundant genera present in this set of samples, other bacteria previously reported to colonize the reproductive tract such as *Bifidobacterium*, *Megasphaera*, *Parvimonas*, *Prevotella*, *Propionibacterium*, and *Veillonella* were found (Figure 5, A).[30,31] The microbiome results using NGS were concordant with RT-PCR in 11 of 12 cases and coincided with the microbial culture in 9 of 12 cases, showing an accuracy of 91.67% and 75%, respectively (Table 3). Interestingly, in 2 cases in which the RT-PCR was negative and culture method was positive (cases 17 and 26), the taxonomic assignment obtained in the microbiota confirmed the results of the RT-PCR, with no detection of bacterial DNA of the pathogens isolated in the microbial culture (Figure 5, B). To better clarify this, the percentage of lactobacilli in these samples was also considered, based on previous reports demonstrating that a percentage of *Lactobacillus* species in the uterine cavity are associated with a healthy endometrial status.[31] This analysis showed a low percentage of lactobacilli in all those samples with a positive diagnosis of chronic endometritis by all

TABLE 2
Results of chronic endometritis diagnosis by molecular and classic methods

Patient	RT-PCR	Histology	Hysteroscopy	Microbial culture
1	*Enterococcus* species, *Staphylococcus* species	Negative	Positive	Negative
2	*Gardnerella vaginalis*	Negative	Positive	Negative
3	Negative	Positive	Positive	Negative
4	Negative	Negative	Positive	Negative
5	*Gardnerella vaginalis*	Negative	Positive	Negative
6	Negative	Negative	Positive	Negative
7	Negative	Negative	Positive	*Enterococcus faecalis*
8	*Streptococcus* species, *Gardnerella vaginalis*	Positive	Positive	*Streptococcus agalactiae*
9	*Streptococcus* species	Negative	Positive	*Enterococcus faecalis*
10	Negative	Negative	Negative	Negative
11	*Streptococcus* species	Negative	Positive	*Streptococcus agalactiae*
12	*Streptococcus* species	Negative	Positive	*Staphylococcus epidermidis*, *Ureaplasma*
13	*Staphylococcus* species	Positive	Positive	Negative
14	*Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	*Streptococcus agalactiae*, *Ureaplasma*
15	*Gardnerella vaginalis*, *Escherichia coli*	Positive	Positive	*Escherichia coli*
16	Negative	Negative	Positive	*Enterococcus faecalis*, *Escherichia coli*
17	Negative	Positive	Positive	*Enterococcus faecalis*, *Ureaplasma*
18	*Streptococcus* species	Positive	Positive	*Streptococcus agalactiae*
19	*Streptococcus* species	Positive	Positive	*Escherichia coli*
20	*Streptococcus* species, *Enterococcus* species	Negative	Positive	*Ureaplasma*
21	*Streptococcus* species, *Enterococcus* species, *Escherichia coli*	Negative	Positive	*Enterococcus faecalis*
22	Negative	Positive	Positive	Negative
23	*Streptococcus* species, *Escherichia coli*, *Klebsiella pneumoniae*	Positive	Positive	Negative
24	*Klebsiella pneumoniae*	Positive	Positive	*Ureaplasma*
25	*Streptococcus* species	Negative	Positive	Negative
26	Negative	Positive	Positive	*Enterococcus faecium*
27	Negative	Negative	Positive	Negative
28	Negative	Negative	Positive	*Staphylococcus epidermidis*
29	Negative	Positive	Positive	Negative
30	*Enterococcus* species	Positive	Positive	*Enterococcus faecalis*, *Streptococcus mitis*
31	*Streptococcus* species	Positive	Positive	*Klebsiella pneumoniae*
32	Negative	Positive	Positive	Negative
33	Negative	Negative	Positive	*Staphylococcus aureus*
34	Negative	Positive	Positive	Negative
35	Negative	Positive	Positive	*Staphylococcus aureus*
36	*Mycoplasma hominis*	Negative	Positive	Negative

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TABLE 2
Results of chronic endometritis diagnosis by molecular and classic methods *(continued)*

Patient	RT-PCR	Histology	Hysteroscopy	Microbial culture
37	*Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	Negative
38	Negative	Negative	Positive	*Ureaplasma*
39	*Streptococcus* species	Positive	Positive	*Streptococcus agalactiae*
40	Negative	Negative	Positive	*Enterococcus gallinarum*
41	*Streptococcus* species, *Escherichia coli*	Positive	Positive	Negative
42	*Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	Negative
43	*Enterococcus* species, *Enterobacteriaceae*, *Escherichia coli*	Negative	Positive	*Staphylococcus aureus*, *Ureaplasma*
44	*Enterococcus* species, *Streptococcus* species	Positive	Positive	*Escherichia coli*
45	*Streptococcus* species	Positive	Positive	Negative
46	*Enterococcus* species, *Streptococcus* species, *Escherichia coli*	Negative	Positive	*Enterococcus faecalis*
47	*Enterococcus* species, *Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	Negative
48	*Streptococcus* species, *Klebsiella pneumoniae*	Negative	Positive	*Candida albicans*, *Ureaplasma*
49	Negative	Negative	Positive	Negative
50	*Streptococcus* species	Negative	Positive	*Escherichia coli*
51	Negative	Positive	Positive	Negative
52	*Enterococcus* species, *Streptococcus* species	Positive	Positive	Negative
53	*Streptococcus* species	Negative	Positive	Negative
54	*Streptococcus* species	Negative	Positive	Negative
55	Negative	Negative	Positive	Negative
56	Negative	Positive	Positive	Negative
57	Negative	Negative	Positive	*Enterococcus faecalis*
58	Negative	Negative	Positive	Negative
59	Enterobacteria	Negative	Positive	Negative
60	*Streptococcus* species, *Escherichia coli*	Negative	Positive	*Streptococcus agalactiae*
61	Negative	Negative	Positive	*Staphylococcus aureus*, *Ureaplasma parvum*
62	Negative	Negative	Positive	*Streptococcus gallolyticus*
63	*Streptococcus* species	Negative	Negative	*Ureaplasma*
64	Negative	Positive	Positive	Negative
65	*Streptococcus* species	Negative	Positive	*Streptococcus agalactiae*

PCR, polymerase chain reaction; *RT*, real-time.
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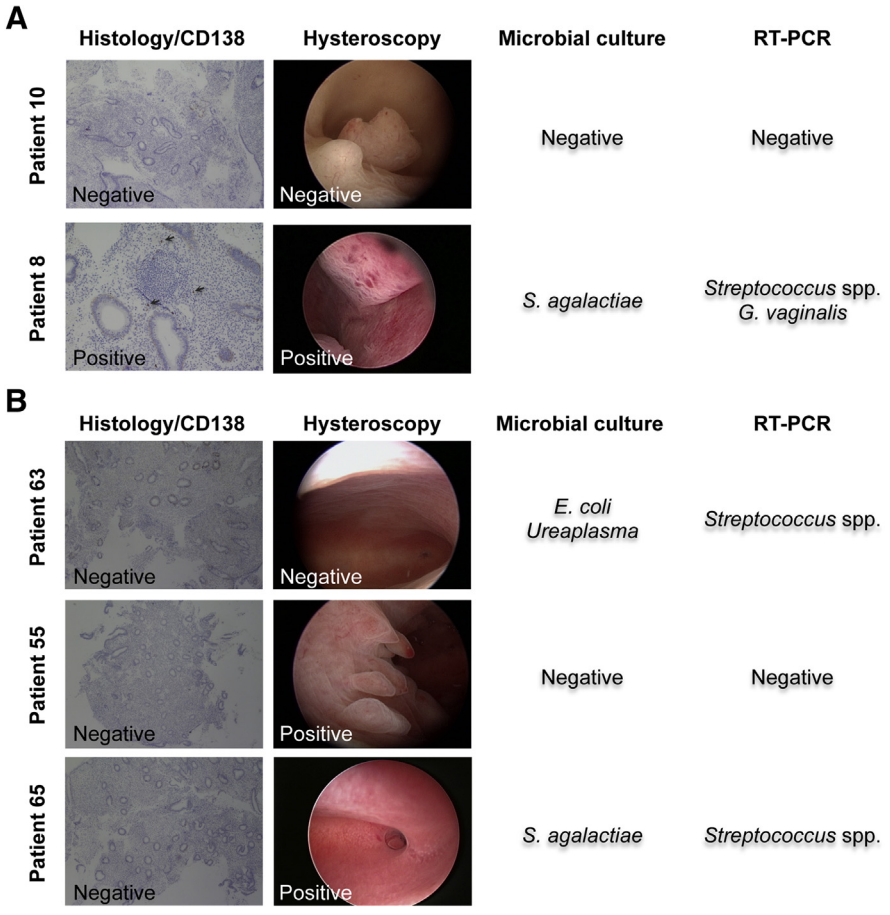
the methods analyzed, with the exception of 1 sample with 93.2% of *Lactobacillus* and a very low percentage of the pathogens detected by either microbial culture or RT-PCR (case 30). Moreover, in the 2 cases (cases 8 and 15) in which we detected *G. vaginalis* by RT-PCR, the sequencing results confirmed its presence in the samples (Figure 5).

By contrast, a high percentage of lactobacilli was detected in the samples that proved negative for the molecular diagnosis of chronic endometritis (cases 10, 17, 26, and 35), supporting the results previously obtained from the RT-PCR analysis (Figure 5, A).

Finally, sensitivity, specificity, positive and negative predictive values, and

FIGURE 4
Histological and hysteroscopic diagnosis of chronic endometritis



A, Concordant chronic endometritis results in patients/samples analyzed by 4 methods compared in this study (3 classic methods and real-time [RT] polymerase chain reaction method [PCR]). **B**, Discordant chronic endometritis results in patients/samples analyzed by 4 methods compared in this study. Black arrows show $CD138^{+}$ cells.

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false-positive and -negative rates were assessed for each individual classic method and their combination in comparison to the RT-PCR diagnosis (Table 4), with the best results obtained when both RT-PCR and NGS diagnosis of chronic endometritis was compared to the 3 concordant classic methods.

Molecular microbiology in patients without chronic endometritis

From the 65 samples analyzed for the 3 classic methods, only 1 presented concordant negative results for all the techniques. Then, to evaluate the efficacy of the RT-PCR method to detect negative cases of chronic endometritis, endometrial samples from 10 control subjects, that proved negative for histology and microbial culture, were subjected to the molecular method. The results of these samples showed negative results for the 9 pathogenic bacteria tested (Table 5) consistent with their negative results for the classic methods analyzed. However, RT-PCR was able to detect very small amounts of DNA from *G. vaginalis* (case C2) and *M. hominis* (case C5), but their levels were below the threshold of positive controls. These data support the efficacy of molecular microbiology to discriminate between positive and negative cases of chronic endometritis, based on the detection of 9 pathogens usually causing the disease.

Comment

Surprisingly, diagnosis of chronic endometritis still depends upon the method used. The current diagnostic gold standard for chronic endometritis is histological examination although is subjective based on different criteria depending on the authors, while hysteroscopy and endometrial culture present controversial reliability in the diagnosis of such condition. Whether histological examination of endometrial tissue, hysteroscopic observation of the uterine cavity, or microbial culture are used has a significant impact on diagnostic accuracy and the different techniques applied to the same patient/sample may yield contradictory results and misleading conclusions (Figure 4, B). The concordant results among the 3 classic techniques in the diagnosis of chronic endometritis in our study was only 20%. Histology and hysteroscopy are highly subjective, unspecific, and rely on the individual observations of the pathologist or endoscopic surgeon.[12] Also, they cannot discriminate the causal agent of chronic endometritis, which in turn leads to broad-range therapy and likely results in recurrent chronic endometritis. Unlike them, microbial culture is able to identify the culturable microorganisms present in the endometrial tissue. However, some chronic endometritis-causing bacteria such as *Ureaplasma* species, *M. hominis*, and *G. vaginalis* are not culturable in standard laboratory conditions, which leads to false-negative results and/or contamination bias. For the aforementioned reasons, a reliable method for the diagnosis of chronic endometritis based on molecular detection and quantification of DNA from pathogens present in the endometrium is needed to improve clinical management.

In modern medicine, nucleic acid amplification tests that are highly sensitive and specific, and in some cases minimally invasive, are recommended to screen for infectious diseases in men and women.[32] Neither histology nor

TABLE 3
Microbiota profile of endometrial samples by 16S ribosomal RNA gene sequencing

			16s rRNA sequencing													
Patient	Microbial culture	RT-PCR	*Lactobacillus* , %	*Enterococcus* , %	*Staphylococcus* , %	*Streptococcus* , %	*Mycoplasma* , %	*Enterobacteriaceae* , %	*Escherichia* , %	*Klebsiella* , %	*Gardnerella* , %	*Ureaplasma* , %	*Chlamydia* , %	*Neisseria* , %	No. of mapped reads	Alpha diversity (Shannon index)
8	*Streptococcus agalactiae*	*Streptococcus* species, *Gardnerella vaginalis*	13.75	2.52	4.46	30.87	0.00	1.00	0.00	0.00	1.48	0.00	0.00	0.00	121,624	7.32
10	Negative	Negative	99.94	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	218,076	2.94
15	*Escherichia coli*	*Gardnerella vaginalis*, *Escherichia coli*	74.40	0.36	1.97	0.68	0.02	1.00	0.00	0.00	1.34	0.00	0.00	0.00	96,697	6.12
17	*Enterococcus faecalis*, *Ureaplasma*	Negative	96.49	0.00	0.11	0.11	0.00	0.30	0.00	0.00	0.43	0.00	0.00	0.00	62,033	3.79
18	*Streptococcus agalactiae*	*Streptococcus* species	0.32	0.00	0.25	81.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	102,443	3.63
19	*Escherichia coli*	*Streptococcus* species	69.21	0.03	2.65	1.06	0.03	3.00	0.00	0.02	2.39	0.01	0.00	0.00	231,538	7.78
24	*Ureaplasma*	*Klebsiella pneumoniae*	ND													
26	*Enterococcus faecium*	Negative	98.75	0.00	0.11	0.25	0.00	0.02	0.00	0.00	0.04	0.00	0.00	0.00	207,848	3.41
30	*Enterococcus faecalis*, *Streptococcus mitis*	*Enterococcus* species	95.21	0.17	0.05	0.04	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	27,178	4.67
31	*Klebsiella pneumoniae*	*Streptococcus* species	6.07	0.00	7.93	1.91	0.00	3.00	0.00	1.25	0.00	0.00	0.00	0.00	36,673	8.03
35	*Staphylococcus aureus*	Negative	91.54	0.00	0.10	0.69	0.00	0.02	0.00	0.00	7.18	0.00	0.00	0.00	149,621	4.14
39	*Streptococcus agalactiae*	*Streptococcus* species	1.33	0.00	0.04	97.54	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	91,595	2.88
44	*Escherichia coli*	*Enterococcus* species, *Streptococcus* species	1.03	0.00	0.12	6.49	0.00	0.01	0.00	0.00	91.39	0.00	0.00	0.00	177,273	4.06

ND, not determined; *PCR*, polymerase chain reaction; *rRNA*, ribosomal RNA; *RT*, real-time.

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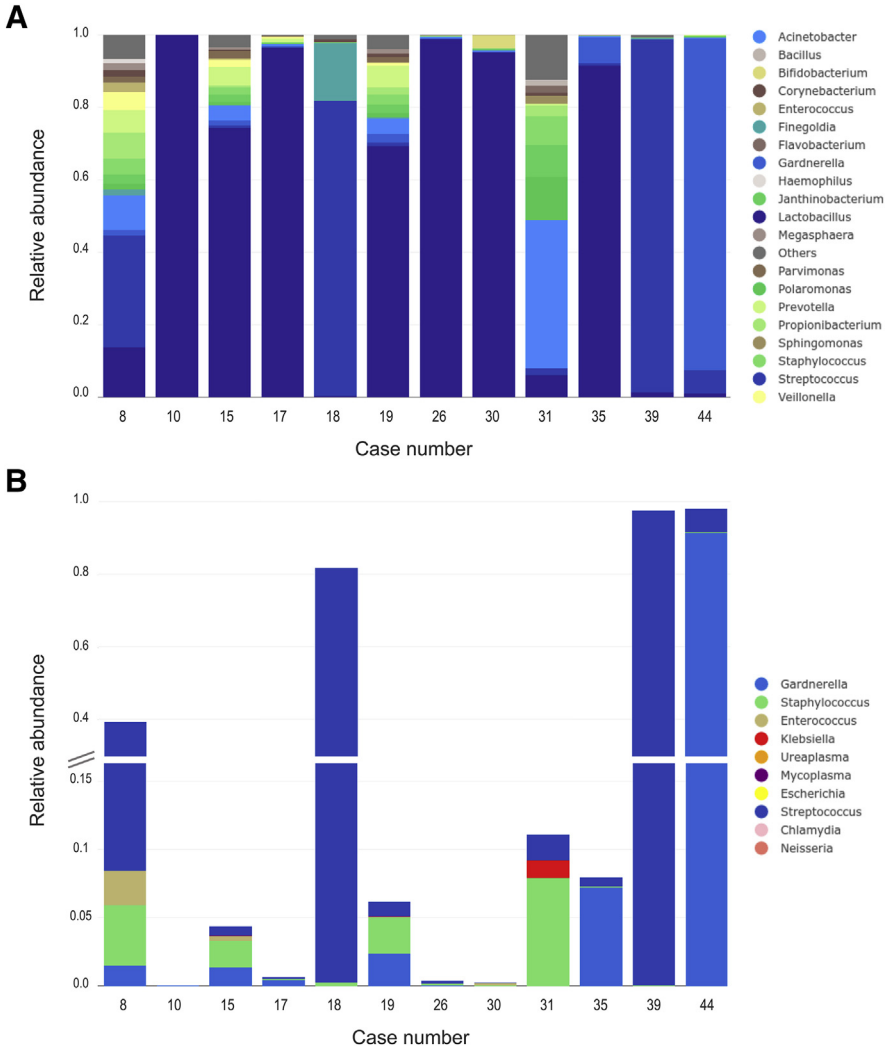
FIGURE 5
Endometrial microbiome assessed by 16S ribosomal RNA gene sequencing



A, Microbiome composition profiles showing 20 most-abundant genera and their relative abundances in endometrial samples of patients with consistent chronic endometritis (CE) diagnosis by 3 classic methods. **B**, Relative abundance of most frequent bacteria causing CE: *Ureaplasma*, *Neisseria*, *Chlamydia*, *Staphylococcus*, *Streptococcus*, *Gardnerella*, *Enterococcus*, *Klebsiella*, *Escherichia*, and *Mycoplasma* in endometrial samples of patients with consistent CE diagnosis by 3 classic methods.

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endoscopy are being used as part of routine diagnosis for infectious diseases in organs and/or tissues except for the uterine cavity. Chronic endometritis is underdiagnosed and thus rarely considered, although clinically relevant in reproductive medicine because it has been associated with repeated implantation failure and recurrent pregnancy loss.[17,20,21,33-35] The high prevalence of common bacteria is not surprising considering that 60% of women with pelvic inflammatory disease have nongonococcal/nonchlamydial infection.[36] Our panel for molecular microbiology was selected to cover >80% of clinical cases of chronic endometritis. The bacteria primarily responsible for the disease are streptococci, *E. faecalis*, *E. coli*, and staphylococci, accounting for 38.5%, 19.3%, 16.3%, and 5.9% of cases, respectively.[3] Recently, dysbiosis of the endometrial cavity has been associated with poor reproductive outcomes in assisted reproductive treatment patients, suggesting that pathogenic deviations of the *Lactobacillus* endometrial content (a signature of chronic endometritis) could play a role in infertility.[31]

Principal findings of the study

Our study demonstrates the usefulness of a simple RT-PCR test for the molecular diagnosis of chronic endometritis in endometrial samples using a comprehensive panel of primers to detect the most common microorganisms involved.

Our results show that RT-PCR is a robust and specific technique able to discriminate targeted bacterial DNA even in challenging conditions such as frozen endometrial biopsies. The molecular analysis had a degree of agreement of 76.92% when endometrial samples showed concordant results by all 3 classic methods. In this subset of 13 samples, molecular microbiology had 75% sensitivity and 100% specificity compared to concordant histology + hysteroscopy + microbial culture diagnosis (Table 4). Among them, *Streptococcus* species were the most abundant bacteria detected (47%), followed by *Enterococcus* species (15%), *E. coli* (12%), *K. pneumoniae* (5%), *Staphylococcus* species (3%), and *M. hominis* (2%); these findings are consistent with previously reported microbial culture data.[3] Interestingly, *G. vaginalis*, a reproductive tract pathogen usually neglected in chronic endometritis diagnosis, was detected in 7% of the samples analyzed using the molecular method. In contrast, *C. trachomatis* and *N. gonorrhoeae* were undetectable in all tested samples, which is in agreement with other works describing a limited role for *C. trachomatis* and sexually transmitted infection pathogens in the origin of chronic endometritis.[37]

Clinical implications of the study

Single use of classic chronic endometritis diagnostic techniques (histology, hysteroscopy, or microbial culture) showed poor diagnostic accuracies (46.15%, 58.46%, and 66.15%, respectively)

TABLE 4
Molecular diagnosis compared to classic diagnostic methods of chronic endometritis

	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)
Histology (n = 65)	56.00	40.00	46.15	36.84	59.26	60.00	44.00
Hysteroscopy (n = 65)	58.73	50.00	58.46	97.37	3.70	50.00	41.27
Microbial culture (n = 65)	71.43	56.67	66.15	65.79	62.96	43.33	28.57
Histology + hysteroscopy (concordant results, n = 27)	56.00	50.00	55.56	93.33	8.33	50.00	44.00
Histology + hysteroscopy + microbial culture (concordant results, n = 13)	75.00	100.00	76.92	100.00	25.00	0.00	25.00

FNR, false-negative rate; *FPR*, false-positive rate; *NPV*, negative predictive value; *PPV*, positive predictive value.
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(Table 4). Moreover, when the overall results were analyzed in detail, we observed that histology and hysteroscopy, the 2 most subjective methods for chronic endometritis diagnosis, yielded contradictory results in 58.46% of cases (Figure 1 and Table 2). In all the inconsistent cases, histology showed negative results while hysteroscopy showed positive results for the same patient, confirming that, compared to other methods, histopathological evaluation usually underdiagnoses chronic endometritis, and hysteroscopy overdiagnoses. The main limitations of histology result from: (1) dependence on the piece of endometrial sample analyzed, (2) variability of staining, (3) observer experience, (4) phase of the menstrual cycle in which the sample was collected,[38] and (5) unknown clinical relevance of a small amount of plasma cells (below the positive threshold) in the endometrium. In this regard, RT-PCR may be useful for the detection of intrauterine germs when histology is negative, as well as for the decision of a target therapy when histology is positive. Hysteroscopic diagnosis is based on the presence of hyperemia, edema, and/or micropolyps, all of which are endometrial symptoms that could be due to other pathophysiological conditions or a noninfectious inflammation of the uterine cavity, as observed in case 55 that was negative for all the methods used except for hysteroscopy in which the only sign of chronic endometritis was the presence of micropolyps (Figure 4, B). Microbial culture, the most reliable of the 3 classic methods, also presents some limitations mainly represented by

TABLE 5
Molecular diagnosis of endometrial pathogens in negative cases

Subject	Surgery indication/treatment	RT-PCR	Histology	Microbial culture
C1	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C2	Oophorectomy (dermoid cyst); usual technique with Novak	Negative (low detection of *Gardnerella vaginalis*)	Negative	Negative
C3	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C4	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C5	Myomectomy; usual technique with Novak	Negative (low detection of *Mycoplasma hominis*)	Negative	Negative
C6	Myomectomy; usual technique with Novak	Negative	Negative	Negative
T1	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T2	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T3	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T4	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative

PCR, polymerase chain reaction; *RT*, real-time.
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contamination of the microbial culture with skin or environmental bacteria (ie, *S. epidermidis*) and the inability to grow and isolate nonculturable bacteria. The limitations of chronic endometritis diagnosis using individual classic techniques and their misleading results are evident in this study in which only 13 of 65 (20%) of the samples/patients analyzed presented concordant results using all 3 diagnostic methods (Figure 1 and Table 2).

Strengths and limitations

These results indicate that molecular microbiology provides similar results to using the 3 classic methods together, overcoming the bias of using any of the classic methods alone. Other advantages of the molecular method are: (1) RT-PCR is highly sensitive and can identify and quantify very small amounts of bacterial DNA, regardless of their culturable or nonculturable nature, and may be useful in estimating the severity of chronic endometritis; (2) it quantifies bacterial DNA instead of live bacteria, so it is able to detect chronic endometritis pathogens in frozen or fixed samples, facilitating sample collection and storage while avoiding contamination during sample transportation/manipulation;[27] and (3) RT-PCR is a rapid assay that has comparable results to bacterial culture with turnaround times of hours rather than days.[39]

While the results of this study show that the molecular detection of bacterial DNA in endometrial samples is a reliable method for the diagnosis of chronic endometritis, with a degree of agreement of 76.92%, several limitations must be addressed to improve the validity of this molecular test. The main limitation of molecular microbiology is the relatively low negative predictive value, estimated at 25%, compared to concordant histology+ hysteroscopy + microbiology. This could be biased by the vast amount of positive chronic endometritis cases determined by hysteroscopy, as the negative predictive value of the molecular method compared to histology or microbial culture alone was 59% and 63%, respectively. Also, molecular methods do not guarantee that DNA comes from viable bacteria, and the establishment of the minimum amount of bacterial DNA that causes the disease should be determined, as the presence of such DNA in some women could be innocuous depending on the host response to those pathogens and would not discriminate between acute and chronic endometritis. The copy numbers for the interrogated gene could be variable between different bacterial genera/species and could cause a slight deviation on the number of bacteria estimated by either RT-PCR or 16S rRNA NGS. Also, the topological localization of bacterial growth should be considered; for example, *G. vaginalis* forms biofilms at the apical surface of the epithelial layer.[40] This could explain the differential detection of *G. vaginalis* by the 2 molecular methods obtained in patients 35 and 44, which may depend on the presence of bacterial biofilm on the specific sample of tissue analyzed (Table 3). To overcome these limitations, future work could address the technical and clinical improvement of this molecular tool by increasing the number of microorganisms included in the panel, as well as the transition to molecular microbiology diagnosis of chronic endometritis in endometrial fluid samples for minimally invasive detection of this disease.

Conclusion

In conclusion, this study demonstrates that compared to the commonly used hysteroscopy, histology, or microbial culture diagnostic methods, RT-PCR effectively detects and quantifies bacterial DNA from chronic endometritis-causing pathogens in endometrial samples providing a feasible, faster, and cheaper method for the diagnosis of chronic endometritis.

Furthermore, the microbiome results using NGS were concordant with RT-PCR in 91.67% of cases and coincide with the microbial culture in 75% of samples because it allows for the detection of both culturable and nonculturable bacteria. The high sensitivity of molecular microbiology allows for the detection of endometrial colonization in patients without histological signs of chronic endometritis, providing additional information to improve the current detection of this invisible endometrial pathology in asymptomatic infertile patients. ■

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