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FULL PAPER

Comparison between a novel molecular tool and conventional methods for diagnostic classification of bacterial vaginosis: is integration of the two approaches necessary for a better evaluation?

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SUMMARY

The etiological cause of bacterial vaginosis (BV) is the change of the vaginal ecosystem characterized by a decrease of lactobacilli and an increase of other germs, such as *Gardnerella vaginalis* and *Atopobium vaginae*. Molecular tools have revolutionized the diagnosis of these conditions. The aim of this paper was to compare results obtained from 158 vaginal swabs collected from women aged between 18 and 59 years old and subjected to microscopic evaluation (Nugent Score), culture and to the multiparametric molecular assay Vaginitis and Vaginosis Multiplex-Tandem (MT) PCR (AU27117) - Nuclear Laser Medicine. In 50 samples we also used matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for bacterial microbiome identification.
Our results showed a moderate concordance between traditional and molecular methods for diagnosis of candidiasis and a lower concordance for BV and normal flora. MALDI TOF MS allowed us to discriminate more than 10 species of lactobacilli with a greater abundance of *Lactobacillus gasseri*, *Lactobacillus paracasei* spp. *paracasei*, *Lactobacillus pentosus* and *Lactobacillus crispatus* in BV and altered flora.
This work underlined how the integration of different assays and metagenomics studies can greatly expand our current understanding of vaginal microbial diversity, providing more reliable diagnostic criteria for BV and its intermediate condition diagnosis.

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INTRODUCTION

The most common vaginal infections diagnosed in women of childbearing age and in pregnant women are bacterial vaginosis (BV) and candidiasis (Onderdonk *et al.,* 2016; Paladine and Desai, 2018). The term BV came into use in the early 1980s to indicate the presence of clinical symptoms not associated with a known pathogen (nonspecific vaginitis) and usually without the hallmarks of an acute infectious inflammatory process that includes high levels of polymorphonuclear cells as part of the vaginal discharge, in contrast to other forms of vaginitis caused by specific microorganisms (Spiegel, 1991).
Several epidemiological data confirm that the etiological cause of BV is the change of the vaginal ecosystem characterized by a decrease or loss of certain *Lactobacillus* spp., such as *Lactobacillus crispatus* and *Lactobacillus jensenii* (Fredricks *et al.,* 2005; O'Hanlon *et al.,* 2013; Srinivasan *et al.,* 2012), which are important contributors to the maintenance of the normal vaginal flora, and an increase of anaerobic germs, including *Gardnerella vaginalis* (GV), *Atopobium vaginae* (AV), *Prevotella* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., *Bacteroides* spp., *Clostridiales* etc. Nevertheless, the list of these microorganisms still appears to be on the rise (Onderdonk *et al.,* 2016; Boris and Barbés, 2000; Dactu, 2014). Even if many women are asymptomatic, symptoms associated with BV usually include vaginal itching or burning and production of thin white or grey vaginal discharge with a strong fish-like odour (Sobel, 2000), and if untreated or treated ineffectively, they may incur serious complications, such as urogenital upper tract disorders, preterm delivery, and sexually transmitted infections (Taylor *et al.,* 2013; Bautista *et al.,* 2016; Leitich *et al.,* 2003). For this reason, an accurate diagnosis of BV is critical for appropriate treatment (Schwebke *et al.,* 2018). Diagnosis of BV is traditionally based on both clinical evaluation and microscopic scoring. The diagnosis of BV is clinically confirmed when at least three of the following Amsel criteria show positivity: pH >4.5, presence of a grey and homogeneous discharge, a fishy volatile amine odour in the discharge, especially if treated with a potassium hydroxide solution, and the presence of squamous epithelial cells coated with bacteria (clue cells) when the discharge is examined microscopically (Amsel *et al.,* 1983). At the same time, Nugent scoring is still the gold standard for laborato-

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ry diagnosis of BV and is carried out by the microscopic identification of Gram-stained vaginal smear: the scoring application is based on Gram-positive *Lactobacillus* spp. counting versus Gram-variable coccobacilli, *Gardnerella/Bacteroides* spp., and Gram-negative bacilli (Nugent *et al.,* 1991; Bhat *et al.,* 2011). A Nugent score of 0 to 3 indicates a negative sample, a score of 4 to 6 suggests an altered flora and a score of 7 to 10 indicates a positive sample for BV.

Certainly, the diagnosis of BV based on Nugent score (Nugent *et al.,* 1991) has sub-optimal accuracy because it is operator-dependent. Microscopic examination is laborious and causes subjective uncertainty since the presence of Gram-variable morphotypes can make it difficult. Moreover, a Nugent score from 4 to 6 may indicate the presence of a certain degree of vaginal dysbiosis, that may not necessarily turn into full-blown BV disease and that could simply require a different type of clinical treatment or no treatment at all.

Rapid, accurate and reproducible methods are needed because the problem of laboratory diagnosis of BV often arises from the difficulty of a correct microscopic observation of the vaginal discharge based on the morphological differences of the bacterial species.

In this regard, a molecular assay represents an opportunity to improve the diagnosis of BV (O'Hanlon *et al.,* 2013; Srinivasan *et al.,* 2012; CDC, 2015). In fact, many of the bacterial species involved in the aetiology of BV, such as AV, *Prevotella* spp., and others, can be detected only by amplification tests, because they are not easily grown or are uncultivable (Srinivasan *et al.,* 2012; Gaydos *et al.,* 2017; Lynch *et al.,* 2019).

The aims of the present study were:

1) to compare a semi-quantitative molecular diagnostic test that includes the application of a diagnostic interpretive algorithm to culture and Nugent scoring, in order to overcome their diagnostic subjectivity;
2) to identify alternative diagnostic tools for cases designated as altered or negative by Nugent scoring;
3) to use matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) identification to compare the bacterial microbiome variation in samples analysed by MT-PCR in order to further investigate variability in the vaginal microflora.

MATERIALS AND METHODS

During 2018 we evaluated 158 vaginal swabs collected from different women between 18 and 59 years of age (mean age 38.47±9.13) who came to our attention for various reasons: vulvo-vaginitis symptoms (24.62%) such as vaginal itching and discharge, suspicion of BV (1.26%), fertility disorders (14.56%) or for check-up after therapeutic treatment (1.26%). 4.43% of samples were collected as screening from pregnant women, and a considerable number (53.80%) were analysed as unspecified screening. The exclusion criteria were not-pregnant women with ongoing antibiotic treatments.

All the samples were subjected to Gram stained microscopic evaluation, culture examination and to a multiparametric molecular assay (Vaginitis and Vaginosis MT-PCR-AU27117) on High-Plex 24 System by Nuclear Laser Medicine (NLM). The molecular parameters evaluated were the presence of *T. vaginalis*, *Candida albicans/krusei/glabrata/parapsilosis,* GV, AV and *Lactobacillus iners/crispatus* nucleic acids.

Two nylon-flocked Copan Amies swabs (E-Swabs, Copan) were used to collect mid-vaginal samples; one was used for culture investigations and one was stored at -80°C until batch molecular investigations were performed. An additional dry vaginal swab was collected and immediately used to make a vaginal smear for the Gram staining and microscopic evaluation.

Microscopic evaluation

The microscopic evaluation of the Gram stained vaginal smear was made by a specialist using Nugent's scoring criteria (Nugent *et al.,* 1991).

Culture methods

All the samples were inoculated directly on Chocolate agar + PolyViteX (bioMérieux) and on Modified Thayer Martin Agar (bioMérieux) at 37°C in 5% CO_2 atmosphere, on Columbia agar + 5% sheep blood (COS) (bioMérieux) at 37°C in anaerobiosis and on Sabouraud Gentamicin Chloramphenicol 2 agar (bioMérieux) at 37°C in O_2 atmosphere.

MT-PCR

The High-Plex 24 System, based on the principle of MT-PCR, employs two sequential PCR steps. The first step is a multiplex target amplification that aims to enrich the target content of the sample and that consists of a nested PCR during which the dye that is present in the reaction mix emits fluorescence once it is intercalated in double-stranded DNA. Then, the fluorescence that increases during the following amplification step is monitored and, after the run is complete, is analyzed by the MT Analysis Software (Version 1.7.9) that automatically reports the presence or the absence of the target germs. A human derived DNA and an artificial sequence are used as controls. Through the integration of two parameters (Bacterial balance and Bacterial Load) the software is also able to generate four interpretative comments: normal flora, meaning that a high percentage of lactobacilli has been found; atypical flora, when there are more human cells than bacterial cells or when normal flora is found in very low concentrations (in this condition it is also possible that different species not searched by the MT PCR panel are present); intermediate flora, indicating that there is a greater amount of GA and/or AV but that the bacterial load was not sufficient to indicate BV, and BV.

MALDI-TOF MS

For the 50 samples, all microorganisms grown on agar plates after 2-5 days of incubation with a bacterial load of 10^4 colony forming units (CFU) were identified by MALDI-TOF MS (MALDI Biotyper, Bruker Daltonik GmbH, Bremen, Germany). Three or four homogeneous colonies from each sample were placed on an MSP 96 polished steel target (Bruker Daltonik GmbH, Bremen, Germany) and allowed to dry at room temperature (RT). Each sample spot was overlaid with 1 µL of matrix, which consisted of a saturated solution of α-cyano-4 hydroxycinnamic acid (HCCA) in 50% acetonitrile- and 2.5% trifluoroacetic acid (Sigma-Aldrich) (final concentration 10 mg HCCA/mL) and air-dried at RT. Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik GmbH) using FlexControl software (version 3.0, Bruker Daltonik GmbH). Spectra were recorded in the linear positive mode at a laser frequency of 20 Hz (ion source 1 voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV) within a mass range from 2,000 to 20,137 Da. Each sample was tested in dupli-

cate to assess spectra reproducibility. Two hundred laser shots per sample spot were acquired employing the Flex Control software package. Spectra were internally calibrated by using an Escherichia coli ribosomal proteins BTS (Bacterial Test Standard, Bruker Daltonik GmbH) according to the manufacturer's instructions. All the produced spectra were visually inspected before statistical analysis in BioTyper software (version 3.0; Bruker Daltonik GmbH) that provided the pattern matching with default settings. Results of the pattern-matching process were expressed with MT scores ranging from 0 to 3.0 as proposed by the manufacturer. For each isolate, the highest score of a match against a spectrum in the customized database was used for identification. A score ≥1.7 was used for reliable species identification.

Statistical analysis

Statistical significance was assessed using χ^2 test and a *P*-value of <0.05 was considered significant. The statistical difference for overall percent agreement values between traditional methods and MT-PCR was determined using the Cohen's kappa coefficient (k). A kappa statistic of 0.00-0,20 indicates that there is no agreement, 0.21 to 0.39 indicates minimal agreement, 0.40 to 0.59 weak agreement, 0.60 to 0.79 moderate agreement, 0.80 to 0.90 strong agreement and above 0.90 indicates almost perfect agreement (McHugh, 2012).

RESULTS

Results for the main microorganisms searched by traditional and molecular methods are summarized in *table 1*.

By Nugent scoring we found that 38.6% of samples were normal (0 to 3 Nugent scoring), 15.8% were with BV (7 to 9 Nugent scoring), 9.5% showed signs of altered flora (Nugent scoring 4 to 6), 27.2% showed characteristics of vaginitis (presence of yeast infection with a load $>10^5$ and leukorrhea or trichomoniasis) and 8.9% showed signs of both vaginitis and vaginosis, due to the concurrent presence of candidiasis and clue cells. On the other hand, the interpretative results obtained by MT RealTime PCR Analysis software showed 41.2% of samples with normal flora, 21.5% with atypical flora, 10.1% with vaginitis, 19.6% with BV, 3.8% with signs of both vaginitis and vaginosis, and 3.8% with intermediate flora. *Table 2* shows that a statistically significant difference between results obtained by Nugent scoring and MT-PCR was found only for vaginitis ($p<0.05$), while it is not possible to compare the conditions of intermediate and atypical flora which together amount to 40 samples, because they are specific pictures of vaginal flora identified by the specific software used to evaluate MT-PCR results. Conversely, by NS we found 15 samples reported as "altered flora" that could be associated with the conditions described above.

The statistical difference calculated by Cohen's k for overall percent agreement values between traditional methods and MT-PCR showed that concordance between the two methods was moderate (k=0.75) when evaluated for diagnosis of candidiasis, while it was weak for BV (k=0.52) and for normal flora samples (k=0.57). Minimal concordance was found for the presence of GV (k=0.39), the diagnosis of vaginitis (k=0.22), and the diagnosis of mixed conditions of vaginitis and vaginosis (k=0.26).

Regarding the presence of lactobacilli, we found a minimal association (k=0.28) between the two methods, while no concordance was found between altered flora identified by traditional methods and atypical flora/intermediate flora obtained by MT-PCR (k=0.18).

Overall, there was a higher percentage of BVs identified by MT-PCR than by traditional methods (19.6% vs 15.8%) and normal flora samples were identified mostly by molecular methods (41.1% vs 38.6%). Conversely, a greater amount of mixed conditions (BV + vaginitis) were detected by traditional methods than by MT-PCR (8.9% vs 3.8%).

The ability to identify the presence of GV and/or AV made MT-PCR more accurate for the identification of BV conditions than traditional methods (*Table 1*).

On the other hand, only 44.4% of samples with GV and

Table 1 - *Comparison between results obtained by traditional methods (Nugent score and culture) and MT-PCR for the detection of the most relevant microorganisms.*

Germ	*N. of positive sample by microscopic evaluation (Nugent score)*	*N. of positive sample by MT-PCR*	*p*	*N. of positive sample by culture method*	*N. of positive sample by MT-PCR*	*p*
Candida spp.	30	31	1.00	27	31	0.66
G. vaginalis	32	72	**<0.01**	29	72	**<0.01**
A. vaginae	/	47	-	/	47	-
T. vaginalis	1	2	-	/	2	-
Lactobacillus spp.	120	102	**0.04**	117	102	0.09

Table 2 - *Comparison between the interpretation results obtained by Nugent score and MT-PCR. BV: bacterial vaginosis.*

Interpretation/diagnosis	*N. of positive sample by Nugent score*	*N. of positive sample by MT-PCR*	*p*
Normal flora	61	65	0.73
BV	25	31	0.54
Altered flora	15	/	-
Mixed vaginitis and BV	14	6	0.11
Vaginitis	43	16	**<0.01**
Intermediate flora	/	6	-
Atypical flora	/	34	-
Total samples	158	158	

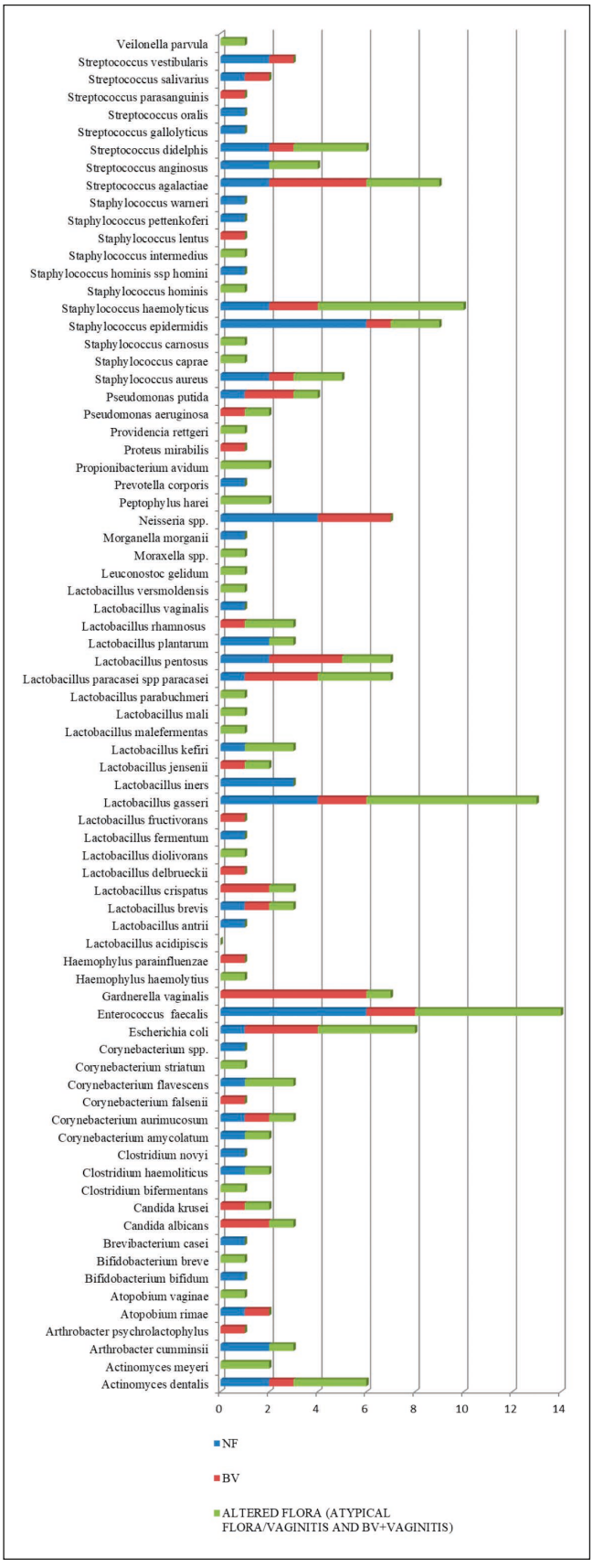

Figure 1 - *Distribution of bacterial species identified by MALDI-TOF MS and isolated from the last consecutive 50 samples. Here they appear in association to MT-PCR interpretative results. NF: normal flora; BV: bacterial vaginosis.*

42.6% samples with AV showed an interpretative result attributable to BV or BV + vaginitis conditions by MT-PCR, while the remaining percentages were associated with intermediate conditions, such as atypical and intermediate flora.

Lastly, we decided to use MALDI TOF to identify the bacterial colonies grown on agar plates with a bacterial load 10^4 in the last 50 consecutive samples (about 32% of the total vaginal swabs collected) in order to highlight the possible presence of relevant bacteria that are not detected by MT-PCR. The results are shown in *Figure 1*, where they appear associated to MT-PCR interpretative results. The figure shows that about 12 taxa were the most abundant in our samples: *Actinomyces dentalis*, *Escherichia coli, Enterococcus faecalis, Lactobacillus gasseri, Lactobacillus paracasei spp paracasei, Lactobacillus pentosus, GV, Neisseria* spp., *Staphylococcus epidermidis, Staphylococcus haemolyticus, Streptococcus agalactiae, Streptococcus didelphi. E. faecalis* was found with the same frequency in normal and altered flora, *L. gasseri* was found more frequently in samples with atypical flora/altered flora. *S. haemolyticus*, *L. paracasei* spp. *paracasei* and *L. pentosus* showed a higher frequency in BV-positive samples; conversely, *Actinomyces* spp. were present only in altered flora, *L. iners* only in normal flora and *L. crispatus* only in BV and altered/atypical flora.

DISCUSSION

Vaginal infections affect women's quality of life by causing frustration, sexual dysfunction and vulvovaginal discomfort. In addition to the direct health care costs associated with the management of vaginal infections, there are also the indirect costs related to adverse reproductive health consequences (Kenyon *et al.*, 2013; Onderdonk *et al.*, 2016). The problem of laboratory diagnosis of BV arises from the difficulty of a correct microscopic observation of vaginal secretion based on the morphological differences of bacterial species. During the past decades, several epidemiological studies have been done about BV prevalence and the risk factors involved in its development. Nevertheless, most of them gave inconclusive results, especially because, even if clinical symptoms associated with the disease are relatively easily measured, not all symptoms occur in all women with BV diagnosis and most BVs are asymptomatic, due to the variability in individual responses to potentially inflammatory mediators produced by the great variety of microorganisms that constitute the vaginal microbiome (Onderdonk *et al.*, 2016). Most recent studies on vaginal microflora are based on the sequencing of the 16S rRNA region (Fredricks et al, 2005; Lamont *et al.*, 2011; Srinivasan and Fredricks, 2008; Aagaard *et al.*, 2012) and show the presence of a large amount of anaerobic and fastidious bacteria that are often uncultivable or not easy to grow (Diopa *et al.*, 2019).

Therefore, a simple, standardized and inexpensive molecular assay able to identify the presence of lactobacilli and other organisms characteristics of a vaginal microbiome at risk for negative health consequences and their abundance represents an opportunity to improve the diagnosis of BV (Friedricks *et al.*, 2007; O'Hanlon *et al.*, 2013, CDC, 2015; Dols *et al.*, 2016; Gaydos *et al.*, 2017).

In this study, we evaluated the analytical performance of Vaginitis and Vaginosis MT-PCR (AU27117) kit for the determination of some of the main microorganisms that favour the pathological states of BV and intermediate vagino-

sis. We found moderate concordance between microscopic examination associated to culture and molecular method regarding the diagnosis of candidiasis, even if MT-PCR showed the advantage of correct detection of candida co-infections (i.e., differentiating between *C. glabrata* and *C. krusei*, which have fluconazole resistance). Conversely, concordance was lower between traditional and molecular tools for BV and normal flora detection, with a higher detection of GV and AV by MT-PCR and more lactobacilli identified by Nugent score. In fact, we were unable to discriminated between lactobacilli species by Nugent score; conversely, associating MALDI TOF MS identification to NS we were able to discriminate part of the lactobacilli grown on agar, allowing us to detect more than 10 species, with a greater abundance of *L. gasseri*, *L. paracasei* spp. *paracasei*, *L. pentosus* and, in lower amount, *L. crispatus* in BV and altered flora samples and, in contrast to literature data, which reported *L. crispatus* and *L. jensenii*, but not *L. iners*, as important contributors to the maintenance of normal vaginal flora that are decreased or lost in BV, we found *L. iners* only in some normal flora samples (Friedricks *et al.,* 2007; O'Hanlon *et al.,* 2013; Srinivasan *et al.,* 2012; Lepargneur, 2016; Hudson *et al.,* 2020; Borgdorff *et al.,* 2016; Edwards *et al.,* 2019; Shipitsyna *et al.,* 2013).

This discordance, such as the minimal association reported regarding the presence of lactobacilli between the two methods compared in this study, may probably be due to the inability of multiparametric molecular assay based on Vaginitis and Vaginosis MT-PCR (AU27117) to discriminate between *L. iners* and *L. crispatus*, which simplify the result as the presence or absence of "both" species.

Moreover, the inability to compare the altered flora conditions identified by traditional methods and atypical flora and intermediate flora detected by MT-PCR is due to the fact that dysbiosis of cervicovaginal microbiota is defined by Nugent scoring as the presence of a high bacterial diversity with a mixture of (facultative) anaerobic bacteria, while the definition of "atypical flora" obtained by MT-PCR, according to the kit manufacturer's interpretive software instructions, indicates a sample with more human cells than bacterial cells or a sample with normal flora in very low concentrations, conditions that could also indicate the presence of different species not searched by the MT-PCR panel and that for this reason could require an additional test for a correct vaginal flora interpretation. On the other hand, the interpretation of intermediate flora by MT-PCR means an increased amount of GV or AV but insufficient bacterial load to indicate BV.

CONCLUSION

Molecular technologies are an important opportunity to make advances in BV diagnosis, offering greater precision in the identification of commensal and potentially pathogenic microorganisms and their semi-quantitative amount in vaginal flora (Dols *et al.,* 2016; Richter *et al.,* 2019) and, at the same time, providing a variety of different clinical pictures that still require in-depth study and close comparison with symptoms and with old methodologies to be fully understood (Hillier, 2005).

Nevertheless, our study emphasises the importance of integrating traditional and molecular tools in order to expand current knowledge of alterations of the vaginal flora and to improve the therapeutic approach to intermediate vaginosis states that could be underestimated or misidentified by using only Nugent scoring, associated or not to culture. In fact, intermediate vaginoses detected with MT-PCR are characterized by high loads of GV and/or AV, which are undetectable by culture and microscopic examination, with large amounts of lactobacilli.

At the same time, due to the great diversity of vaginal microbiota, culture methods may represent an excellent tool if associated in a complementary way to metagenomics (microbial culturomics), taking full advantage of culture conditions thanks to rapid bacterial identification by MALDI TOF MS (Diopa *et al.,* 2019). In fact, the MT-PCR tested in this study shows inability to discriminate some species of bacteria and lactobacilli which could be important for better classification of intermediate BV conditions.

A limitation of this study was that we used the traditional reference method for BV, characterized by high operator-dependent variability in reading and reporting results and by low accuracy due to the presence of microorganisms that are difficult or impossible to grow (Fredricks *et al.,* 2005; Fredricks *et al.,* 2007; Menard *et al.,* 2008). Multicentre studies, involving a major number of samples and reporting the specific symptomatology of each patient, may certainly improve information about the usefulness of molecular methods in both full-blown and intermediate BV stages.

Conflicts of interest

The authors declare no conflict of interest.

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