International Journal of Microbiology and Biotechnology
2021; 6(3): 71-77
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20210603.12
ISSN: 2578-9678 (Print); ISSN: 2578-9686 (Online)

Rapid Quantitative PCR Assay for the Detection of the Three Vaginal Pathogens *Candida*, *Gardnerella* and *Atopobium* as well as the Commensal *Lactobacillus* Genera

Stephanie Bornes[1, *], Olivier Camarès[1], Marylise Paquet-Gachinat[1], Philippe Veisseire[1], Jacques Ravel[2, 3], Caroline Dausset[4], Adrien Nivoliez[4]

[1]University of Clermont Auvergne, INRAE, VetAgro Sup, UMRF, Aurillac, France

[2]Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, USA

[3]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, USA

[4]Department of Research and Development, Biose Industry, Arpajon-Sur-Cère, France

Email address:

stephanie.bornes@uca.fr (S. Bornes), olivier.camares@uca.fr (O. Camarès), marylise.paquet@uca.fr (M. Paquet-Gachinat), philippe.veisseire@uca.fr (P. Veisseire), jravel@som.umaryland.edu (J. Ravel), c.dausset@biose.com (C. Dausset), a.nivoliez@biose.com (A. Nivoliez)

[*]Corresponding author

To cite this article:

Stephanie Bornes, Olivier Camarès, Marylise Paquet-Gachinat, Philippe Veisseire, Jacques Ravel, Caroline Dausset, Adrien Nivoliez. Rapid Quantitative PCR Assay for the Detection of the Three Vaginal Pathogens *Candida*, *Gardnerella* and *Atopobium* as well as the Commensal *Lactobacillus* Genera. *International Journal of Microbiology and Biotechnology*. Vol. 6, No. 3, 2021, pp. 71-77.
doi: 10.11648/j.ijmb.20210603.12

Received: July 12, 2021; **Accepted**: July 27, 2021; **Published**: August 4, 2021

Abstract: The vaginal microbiota balance is quite fragile and susceptible to the development of vaginosis and candidiasis. The current diagnostic method for bacterial vaginosis relies on the evaluation of different bacterial morphotypes using the Nugent score. This method is only partially in correlation with a DNA sequencing-based diagnostic or Amsel criteria used by clinicians, suggesting the need for new molecular approaches dedicated to the diagnosis of BV. The objective of this study was to develop and validate a quantitative polymerase chain reaction (qPCR) assay for the specific and rapid detection of three vaginal pathogens, *i.e. Candida, Gardnerella* and *Atopobium* and the commensal Lactobacillus genera. For this purpose, four oligonucleotide primer pairs were designed and tested to obtain optimal amplification of the four target genera. The qPCR assay was also tested on the non-target genera and on human DNA. The designed primers allowed specific amplification of the target organisms *in vitro* and in clinical vaginal samples. The qPCR assay designed in this study is effective to specifically detect these genera in clinical samples as a molecular technique complementary to the Nugent score. It can be used in epidemiological studies for understanding the role of these pathogens and to follow their abundance in the microbiota in disease processes such as the development of vulvovaginal candidiasis and bacterial vaginosis.

Keywords: *Lactobacillus*, *Atopobium*, *Gardnerella*, *Candida*, Quantitative PCR, Vaginal Infections

1. Introduction

Lactobacillus spp. are a major component of the vaginal microbiota [1, 2] These bacteria play an important role in preventing colonization by undesirable organisms by exclusion, modulation of host immune response or production of various antibacterial compounds such as lactic acid or antimicrobial peptides [3, 4]. Among this genus, *Lactobacillus jensenii, L. gasseri, L. crispatus* and *L. iners* are the most common species encountered in the vaginal ecosystem [5]. *L. vaginalis, L. acidophilus, L. rhamnosus*, *L. ruminis* and even *Bifidobacterium* spp. have also been identified in lower abundance in vaginal microbiota [6, 5, 7]. The vaginal microbiota balance is quite fragile and susceptible to the development of vaginosis and candidiasis. *Candida albicans,* which is detected in about 20% of women, has been shown to be responsible for 80-90% of vulvovaginal

candidiasis (VVC) [8, 9]. *Gardnerella vaginalis* is present in 100 % of women with bacterial vaginosis (BV) [10]. *Atopobium vaginae*, is associated with 80% of BV cases. It is the strongest bacterial predictor of treatment failure and BV recurrence [11, 10]. *A. vaginae* is often detected in symptomatic BV together with *G. vaginalis* in the biofilm adherent to the vaginal mucosa [12]. *A. vaginae* is believed to be dependent on *G. vaginalis* to colonize the vaginal epithelium. However *A. vaginae* is often predominant in advanced stages of the condition [13]. The current diagnostic method for BV in research setting relies on the evaluation of different bacterial morphotypes on a Gram-stained vaginal smear, known as the Nugent score [14]. This method fails to differentiate a normal from an abnormal vaginal microbiota in a significant proportion of women, suggesting the need for new molecular approaches dedicated to the diagnosis of BV. The BV diagnostic using Nugent score is only partially in correlation with a DNA sequencing-based diagnostic. Moreover, there is a difference in sensibility and specificity of this test comparing to the Amsel criteria used by clinicians; Amsel-BV-positive samples are not all Nugent-BV or molecular-BV positive [15]. To address these limitations, there is a need to quantify key genera or species in the vaginal microbiota using complementary molecular approaches. Quantitative PCR technology represents a precise, quantitative, more affordable and faster than sequencing-based approaches that could be used in research and clinical trials. Such test would allow the rapid detection of early signs of transition towards BV state as well as BV remission after preventive and curative therapies, including the live biotherapeutic products (LBP). The growing number of vaginal bacterial genome sequences available required that previously published primers be revised for increased accuracy. In this work, we designed primers that specifically target the three major vaginal genera associated with bacterial vaginosis and non-optimal vaginal microbiota as well as *Lactobacillus,* which is associated with optimal vaginal microbiota. These primers were checked for specificity against DNA extracted from *in vitro* microbial cultures (single strain or coculture) as well as DNA extracted from human vaginal samples collected as part of the UMB-HMP study and for which the composition of the vaginal microbiota had already been established [16].

2. Materials and Methods

2.1. Bacterial, Fungal Strains and Human Epithelial Cells

Lactobacillus acidophilus, L. brevis, L. buchneri, L. casei, L. crispatus, L. delbrueckii, L. gasseri, L. jensenii, L. rhamnosus, L. reuteri, L. vaginalis and *L. zeae* from our own culture collection, were grown (inoculated at 1%) in Man, Rogosa, Sharpe (MRS) broth (AES, Bruz, France) at 37°C for 48 h, without shaking. *Candida albicans* ATCC10231 and *C. albicans* clinical strain, *C. glabrata* and *C. tropicalis* were grown in Sabouraud medium (AES, Kerlann, France) at 25°C for 48 h. *Gardnerella vaginalis* ATCC14018 and *Atopobium vaginae* CIP106431^T were grown in brain heart infusion (BHI) (SIGMA, St-Quentin-Fallavier, France) supplemented with yeast extract (1% w/v) (SIGMA, St-Quentin-Fallavier, France), maltose (0.1% w/v) (SIGMA, St-Quentin-Fallavier, France), glucose (0.1% w/v) (SIGMA, St-Quentin-Fallavier, France), and horse serum (10% v/v) (DUTSCHER, St-Quentin-Fallavier, France) and incubated at 5% CO_2 at 37°C. Human Caco-2 cells were grown in Dulbecco modified Eagle's minimal essential medium (DMEM, LIFE TECHNOLOGIE, Villebon sur Yvette, France) supplemented with 20% inactivated fetal calf serum (LIFE TECHNOLOGIE, Villebon-sur-Yvette, France).

2.2. Human Vaginal Samples

The DNA from vaginal samples analyzed in this study was collected as part of the UMB-HMP study [16] for which the composition of the vaginal microbiota was previously established by sequencing of the V1-V3 regions of the 16S rRNA gene. The samples were selected to represent a range of Nugent Score and diagnosed vulvovaginal candidiasis (VVC).

2.3. Quantification of Cultivable Cells

Ten serial dilutions of culture comprising individual strains or strains mixtures were performed in sterile water. The number of cultivable cells was determined by a plate count method on MRS agar (Biomerieux, Marcy l'Etoile, France) for *Lactobacillus* strains, Sabouraud dextrose agar for *Candida* strains (AES, Kerlann, France), BHI agar for *Gardnerella* and *Atopobium* strains (SIGMA, St-Quentin-Fallavier, France). MRS and BHI plates were incubated at 37°C for 72 h and Sabouraud dextrose plates at 25°C for 120 h. The results were expressed as colony forming units per milliliter ($CFU.mL^{-1}$). The whole experimental design was repeated three times.

2.4. DNA Extraction

Microbial cells (single strain or mixture of two strains) were centrifuged (30 sec at 1000 *g*) and lysed using a Precellys 24 (Bertin Technologie, Rockville, US). DNA extraction was performed using MOBIO UltraClean® Microbial DNA Isolation Kit (OZYME, Montigny-le-Bretonneux, France) according to manufacturer's recommendations.

The Caco-2 cells wells were washed three times with 1 ml of PBS, the cell monolayer was detached by addition of 0.1% Triton X-100 solution (LIFE TECHNOLOGIE, Villebon-sur-Yvette, France). DNA extraction was performed using QIAamp DNA micro kit (Qiagen, Coutaboeuf, France) according to manufacturer's instructions. DNA concentrations were measured using Nanodrop 2000c (ThermoScientific, Wilmington, US).

2.5. Quantitative PCR Primers Development and Validation

qPCR primers targeting the 4 genera *Lactobacillus, Candida, Gardnerella* and *Atopobium* were designed (Table 1) with home-made BioPerl script integrating Primer3 [17, 18]. First, primers specificity was checked *in silico* against the all available genomes of strains of *Lactobacillus, Candida,*

Gardnerella and *Atopobium* using BLASTn [19]. Their specificity was first validated against DNA extracted from pure cultures of 17 strains of our collection composed of 12 strains of *Lactobacillus*, 4 strains of *Candida*, 1 strain of *Gardnerella* and 1 strain of *Atopobium* (Table 2), and human DNA by PCR using HotStarTaq Master Mix kit (QIAGEN, Courtaboeuf, France) and a Mastercycle thermocycler (Eppendorf, Le Pecq, France).

To determine the efficiency of the primers in a more complex mixture of strains, one bacterial strain of each genus *i.e. Candida albicans, Gardnerella vaginalis* and *Atopobium vaginae* (from 10^9 to 10^2 cells.mL^{-1}) was mixed with 10^9 cells.mL^{-1} of *L. crispatus*. The amplification efficiency (E) of each couple of primers was determined by qPCR using a 10X target microbial DNA dilution standard curve. The qPCR mix contained 2.5 µl of 10X diluted DNA standard, 6.375 µl of QuantiFast SYBR Green mastermix, 20 nmol of both forward and reverse primers and 3.125 µl of water. Cycling conditions were 94°C for 15 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec (except that 55°C for 30 sec for *Candida*) and 72°C 60 sec. The slope of the regression curve between the DNA concentrations log values and the average Ct values was used to calculate the primers efficiency using the equation: $E = 0.5\ (10^{(-1/slope)})$ x100. A melting curve analysis (60°C to 95°C) was performed to ensure specificity in the amplification. Moreover, primers specificity was validated for their capacity to uniquely amplify DNA from the targeted genus by quantitative PCR. Specificity during qPCR was considered to be validated when the delta Ct was at least of 10 between the targeted and non-targeted genera.

The quantitative PCR (qPCR) reactions were performed using QuantiFast SYBR Green qPCR kit (QIAGEN, Courtaboeuf, France) and on a Rotor Gene Q instrument (QIAGEN, Courtaboeuf, France). Each sample reaction was repeated at least three times.

Table 1. *qPCR primers.*

Genera	Primers sequences	Amplicon size (bp)	Targeted genes
Lactobacillus Lacticaseibacillus Lactiplantibacillus Lentilactobacillus Levilactobacillus Ligilactobacillus Limosilactobacillus	(forward) CGA TGA GTG CTA GGT GTT GGA (reverse) CAA GAT GTC AAG ACC TGG TAA G	186	16S rRNA gene
Gardnerella	(forward) GCC TGA CGA CTG CAG AGA TGT (reverse) ATT AGC ACC ATG TCA CCA TGA	282	16S rRNA gene
Atopobium	(forward) CCC TAT CCG CTC CTG ATA CC (reverse) CCA AAT ATC TGC GCA TTT CA	83	Ribonuclease P
Candida	(forward) CGG GTG GGA AAT TCG GT (reverse) CAA TGA TCG GTA ACG GGT	252	18S rRNA gene

Table 2. *Specificity of the primers designed was verified on the 17 microbial DNA extracts from pure cultures of strains of our collection composed of 12 strains of Lactobacillus, 4 strains of Candida, 1 strain of Gardnerella and 1 strain of Atopobium.*

Species	Lactobacillus amplification	Candida amplification	Gardnerella amplification	Atopobium amplification
L. acidophilus	+	-	-	-
L. brevis	+	-	-	-
L. buchneri	+	-	-	-
L. casei	+	-	-	-
L. crispatus	+	-	-	-
L. delbrueckii	+	-	-	-
L. jensenii	+	-	-	-
L. rhamnosus	+	-	-	-
L. reuteri	+	-	-	-
L. vaginalis	+	-	-	-
L. zeae	+	-	-	-
C. albicans	-	+	-	-
C. albicans clinical strain	-	+	-	-
C. glabrata	-	+	-	-
C. tropicalis	-	+	-	-
G. vaginalis	-	-	+	-
A. vaginae	-	-	-	+
H. sapiens	-	-	-	-

2.6. Determination of Microbial Detection Threshold Using qPCR

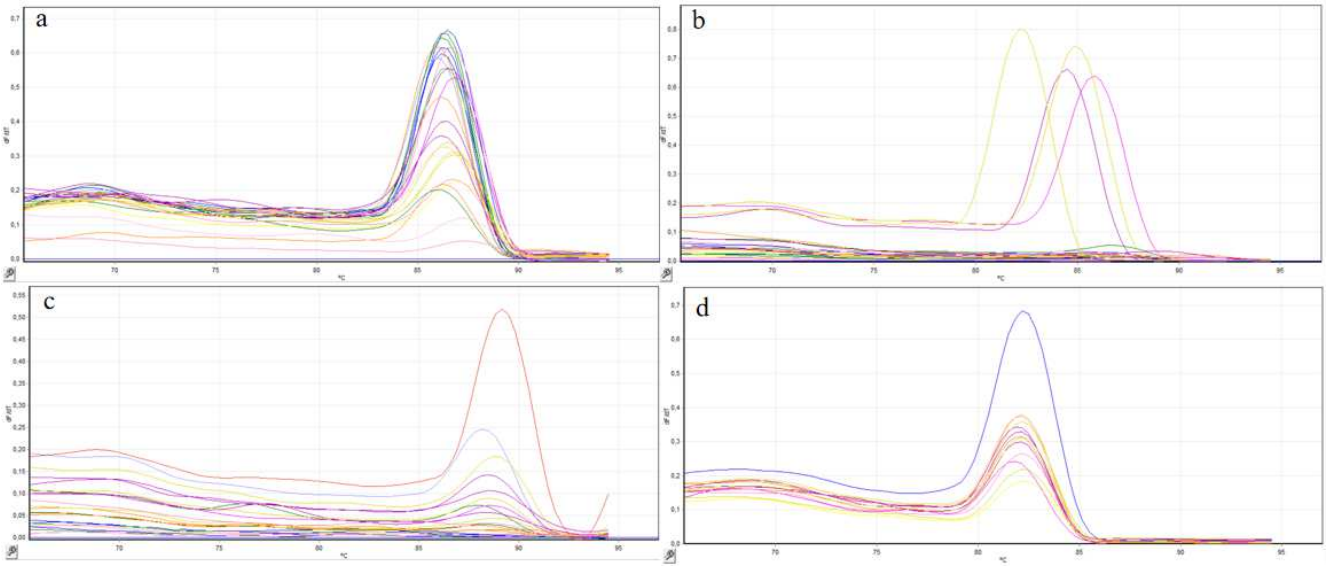
To determine the detection threshold of the pathogen in presence of a physiological population of *Lactobacillus*, 10X serial dilutions of each pathogen microorganism 48 hours-culture were made. Each culture was diluted (10^9 cells.mL^{-1} to 10^3) and mixed with 10^9 cells.mL^{-1} *Lactobacillus crispatus* before quantification of cultivable cells and DNA extraction.

Standard curves were made using DNA extracted from

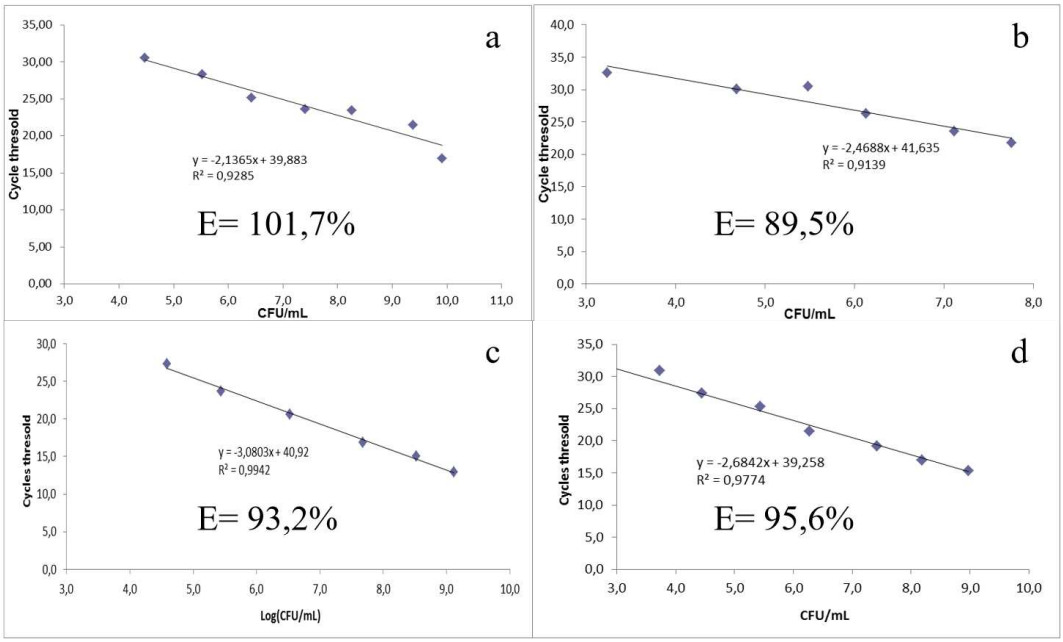
artificial mixtures of microorganisms that included *L. crispatus* with either *Candida albicans* or *Gardnerella vaginalis* or *A. vaginae* 10X dilutions. Standard curves were generated by plotting threshold cycles (Ct) versus bacterial concentration (CFU.mL^{-1}) obtained by a plate count method. The threshold of detection was determined as the highest Ct obtained after serial dilutions except if this Ct value was higher than the no template (NT) control or higher than 30. The quantitative PCR (qPCR) reactions were performed using QuantiFast SYBR Green qPCR kit (QIAGEN, Courtaboeuf, France) and on a Rotor Gene Q instrument (QIAGEN, Courtaboeuf, France). The same experimental conditions than in paragraph 2.5 were used. The experiment was repeated three times.

2.7. Quantification of Pathogenic Strains from Human Vaginal Samples

qPCR experiments were performed using the same experimental conditions than in paragraph 2.5, using DNA extracted from clinical samples collected from patients with or without bacterial vaginosis (BV) established according to the Nugent score [16]. Data generated were analyzed using the QIAGEN Rotor-Gene Q series software 2.1.0.



(A)



(B)

Figure 1. *Quantitative PCR primers validation. (A) Determination of the efficiency of the primers and of their limit of detection using dilutions of Lactobacillus (a), Candida (b), Gardnerella (c) and Atopobium (d). Ten fold serial dilutions of each microorganism were prepared. The graphs show the Cycles threshold (Ct) in function of the initial cell concentration (CFU. mL^{-1}). The primers efficiency (E) is mentioned on the graphs. (B) Determination of the specificity of the primers using the dissociation curves corresponding to the amplification of Lactobacillus (a), Candida (b), Gardnerella (c) and Atopobium (d).*

3. Results

3.1. Quantitative PCR Assay Design and Validation

Four couples of primers specific for the 4 genera *Lactobacillus, Gardnerella*, *Atopobium* and *Candida* were designed. The targeted genes were the 16S rRNA gene for the genera *Lactobacillus* and *Gardnerella,* the 18S rRNA gene for *Candida,* and the Ribonuclease P encoding gene for the genus *Atopobium* (Table 1). Their specificity has been checked using DNA extracted from 12 *Lactobacillus* spp., four *Candida*, one *Gardnerella* and one *Atopobium* strains from our culture collection (Table 2) and from human Caco-2 cells. The fluorescence curves reflecting DNA-SYBR Green interaction showed that the 4 primers pairs designed were specific to their targeted gene (Figure 1A). Concerning the four *Candida* strains tested, a single peak was observed for each strain but the melting temperature was strain-dependent. No amplification was obtained when the assay was conducted with human DNA alone. All the qPCR assay results were in good correlation with our *in silico* specificity prediction. The primers efficiencies were established between 89,5% and 101,7% (Figure 1B).

3.2. Microorganisms Detection Threshold

To establish the threshold of detection for *Candida, Gardnerella* and *Atopobium* in an environment containing *L. crispatus,* mixtures in which CFU counts of each target organism was known were processed. The threshold of detection for *Candida, Gardnerella* and *Atopobium* was calculated according to the standard curves. The threshold of detection for *Candida* was 4.7 Log_{10} $CFU.mL^{-1}$, for *Gardnerella* 3.6 Log_{10} $CFU.mL^{-1}$ and for *Atopobium* 3.5 Log_{10} $CFU.mL^{-1}$, while it was the lowest for *Lactobacillus* at 3.4 Log_{10} $CFU.mL^{-1}$ (Figure 1B).

Table 3. *qPCR detection of Lactobacillus, Candida, Gardnerella and Atopobium genera from human vaginal samples (Log_{10} $CFU.mL^{-1}$) and proportions of the 3 bacterial genera resulting from 16S metabarcoding. Samples under the detection thresold are mentioned as not detected (nd). Samples collected during a Vulvo Vaginal Candidiasis (VVC) event are categorized as DVVC. The 4-day periods just before and just after a VVC, are defined as TBV (transition before VVC) and TAV (transition after VVC), respectively. The time period before VVC is limited to 7 days and defined as BVVC, while all days following the transition after treatment of VVC are defined as AVVC. BV, Bacterial Vaginosis.*

Samples	VVC Status (Nugent score)	BV status (Nugent score)	Log $CFU.mL^{-1}$				16S metabarcoding (%)		
			Lactobacillus	*Candida*	*Gardnerella*	*Atopobium*	*Lactobacillus*	*Gardnerella*	*Atopobium*
1	BVVC	no BV	7,9	nd	7,2	nd	98,68	15,59	0,03
2	DVVC	BV	nd	nd	6,6	nd	76,31	40,26	0,03
3	TAV	BV	4,2	nd	nd	nd	61,84	20,89	6,13
4	BVVC	no BV	5,1	nd	nd	nd	99,94	0,05	0,00
5	DVVC	no BV	6,4	nd	nd	nd	99,71	0,04	0,00
6	BVVC	no BV	7,5	nd	nd	nd	99,99	0,13	0,00
7	DVVC	no BV	5,6	nd	6,7	7,7	99,99	0,05	0,00
8	BVVC	no BV	12,9	10,6	5,3	4,3	99,89	0,01	0,00
9	DVVC	no BV	11,1	6,3	3,8	5,7	99,95	0,00	0,00
10	INT	no BV	12,8	6,0	3,9	nd	99,93	0,00	0,00
11	BVVC	no BV	12,4	5,0	7,0	4,6	99,43	0,53	0,00
12	AVVC	no BV	7,6	nd	nd	nd	99,81	0,01	0,00
13	BVVC	inter-BV	9,6	nd	nd	nd	1,24	0,02	0,04
14	DVVC	no BV	14,0	9,7	8,4	7,3	99,07	8,35	0,15
15	DVVC	inter-BV	9,4	5,6	nd	3,5	0,79	0,00	0,01
16	AVVC	no BV	8,0	nd	nd	nd	0,91	0,00	0,00
17	BVVC	no BV	8,2	nd	6,9	7,0	83,33	5,56	0,00
18	DVVC	no BV	8,6	nd	nd	nd	99,91	0,00	0,00
19	DVVC	no BV	3,6	nd	6,2	7,2	58,13	27,72	0,00
20	AVVC	no BV	5,2	nd	5,5	6,5	56,90	31,73	13,27
21	BVVC	no BV	nd	nd	nd	nd	98,19	0,00	0,00
22	DVVC	inter-BV	6,4	nd	nd	nd	99,84	0,00	0,00
23	AVVC	no BV	5,2	nd	nd	nd	99,83	0,01	0,00
24	TBV	no BV	5,5	nd	nd	nd	98,91	0,00	0,00
25	DVVC	no BV	5,7	nd	nd	nd	99,82	0,00	0,00
26	AVVC	no BV	4,6	5,2	nd	nd	98,71	0,00	0,00

3.3. Microorganisms Detection in Clinical Samples

The applicability of our method in routine diagnostic testing was evaluated using a set of clinical samples. Among these samples, two were defined as « bacterial vaginosis » samples (BV) and three samples as « intermediate bacterial vaginosis » samples (inter BV) and twenty-one samples as « no BV » according to the Nugent score. We applied the qPCR assay to the 26 vaginal samples from patients with VulvoVaginal Candidiasis (VVC) (Table 3). Twenty-four samples out of 26 were *Lactobacillus* positive. The number of *Lactobacillus* varied between a minimum of 3 Log_{10} $CFU.mL^{-1}$ to a maximum of 14 Log_{10} $CFU.mL^{-1}$ of sample. The three inter-BV samples showed a higher level of *Lactobacillus* (between 6.4, 9.4 and 9.6 Log_{10} $CFU.mL^{-1}$) than the two BV samples (3 and 4.2 Log_{10} $CFU.mL^{-1}$). From 4.6 to 14 Log_{10} $CFU.mL^{-1}$ *Lactobacillus* were quantified in the « no BV » samples. The highest levels of *Lactobacillus* were found in no

BV samples. *Candida* was detected in 7 samples out of 26, *Gardnerella* in 11 samples and *Atopobium* in 9 samples. The highest concentration of *Lactobacillus* was associated with a concentration of *Candida* of at least 5 Log_{10} $CFU.mL^{-1}$. In samples in which *Gardnerella* was detected and *Candida* not detected, the level of *Lactobacillus* was low (3 to 5.6 Log_{10} $CFU.mL^{-1}$). With the exception of one sample out of 9, *Atopobium* is co-detected with *Gardnerella*. In the sample containing *Atopobium* (3.5 Log_{10} $CFU.mL^{-1}$) and no *Gardnerella* (sample 15), the level of *Lactobacillus* was high (9.4 Log_{10} $CFU.mL^{-1}$) (Table 3).

Gardnerella was detected in only one of the two « BV » samples and in 10 of the 21 « no BV » samples. The level of *Candida* was low (4.6 and 5.6 Log $CFU.mL^{-1}$) in 2 inter-BV samples, and no detected in the BV ones. Quantitative PCR allowed the detection of the presence of *Candida* in BVVC and DVVC conditions except for 2 samples. In the 5 out of 6 AVVC samples, there is no more *Candida* detected which is in correlation with the expected data of an AVVC treated status. In these 6 samples, there is neither *Gardnerella* nor *Atopobium* detected, except in sample 20.

Moreover, the 26 samples have been tested using metabarcoding sequencing. Some of the data obtained using qPCR were in good correlation with the quantification made from the sequencing of the BV samples. High levels of *Lactobacillus* were quantified in all samples except 3 (samples 13, 15 and 16). *Gardnerella* was quantified in 6 samples; 5 samples out of 6 showed as well high Log_{10} $CFU.mL^{-1}$ *Gardnerella* concentrations. Six percent of *Atopobium* was detected in sample 2 (BV sample) and 13,27% in sample 20 (no BV sample). Concerning the « inter-BV » samples, the quantification of *Lactobacilli* were not in accordance between the 2 technics with very extreme levels (from 0.79 to 99.9%) detected using metabarcoding. The quantification of *Gardnerella* and *Atopobium* using the two approaches was consistent.

4. Discussion

In this work, primers specific to the *Lactobacillus, Candida, Gardnerella* and *Atopobium* genera have been designed. The choice to use genus specific primers to evaluate the *Lactobacilli* population level allowed their use to study the five different Community State Types (CST) defined [5, 20]. We showed that our primers were specific and efficient at detecting the major species of *Lactobacillus*. The primers designed to quantify *Candida, Gardnerella* and *Atopobium* allowed detecting concentrations of 4.5 (*Atopobium*), 4.8 (*Gardnerella*) or 5.1 (*Candida*) log_{10} $CFU.mL^{-1}$. Our qPCR primers would allow the efficient detection of *Candida* species with a sensitivity as low as 10^5 cells per ml and consequently permit to detect a VVC. Surprisingly, in this study, *A. vaginae* was not always detected associated with bacterial vaginosis (1 sample out of 2). The metabarcoding method appeared more sensitive than the qPCR method to detect *A. vaginae* in our BV samples. The presence of *Atopobium vaginae* was associated with *G. vaginalis* on BV and no BV samples, using both technics *i.e.* qPCR and sequencing. Accurate detection of *A. vaginae* is critical as the species has been reported to be involved in therapeutic failures [12]. In our experiments, qPCR appeared to be more sensitive in the detection of *Atopobium* than metabarcoding in most of inter-BV and no BV samples. Nugent score evaluates *Gardnerella* abundance but not *Atopobium vaginae*. Integrating the detection of *A. vaginae* in the score would help for the diagnosis of BV. BV is characterized by a shift in the vaginal ecosystem from the dominant Lactobacillus to a more diversified microbial flora. Since the list of possible agents is increasing, a perspective to make the score still more accurate will be to integrate as well the detection and quantification of other microorganisms including *Mobiluncus*, *Prevotella*, *Megasphaera, Sneathia* and BV-associated bacteria [21].

These two molecular techniques allowed the detection of *Gardnerella* and the data obtained are in quite good correlation. These data suggested that the quantitative PCR assay represents a complementary approach in detecting BV and VVC. Our data suggested that qPCR was reliable as well as 16S rRNA gene sequencing, but was cheaper and less time consuming. Whilst the current laboratory detection method for BV i.e. Gram-stain Nugent score does not give an exhaustive description of the pathogenic vaginal flora and given that these new primers have high specificity in the presence of human DNA, they could be used to develop an improved scoring system for BV. BV is generally defined by high abundance of *Gardnerella* and *Atopobium,* and low abundance of *Lactobacillus* [22, 7], whereas *Candida* infection have been shown to be agnostic to the *Lactobacillus* composition of the vaginal microbiota [23]. Our primers allow the quantitation of *Lactobacillus*, *Candida*, *Gardnerella* and *Atopobium* and thus are able to differentiate VVC, BV and VVC-BV from optimal vaginal microbiota.

5. Conclusion

Our study shows that the designed qPCR primers are sensitive and specific for the rapid detection and quantification of *Latobacillus, Candida*, *Gardnerella* and *Atopobium* as a tool for the rapid evaluation of vaginal microbiota, and ultimately as diagnostic tool for BV and VVC. These primers could also be used to follow the composition of vaginal microbiota during treatment trials and evaluate the therapeutic efficacy of new products, including live biotherapeutics.

Acknowledgements

SB, CD, PV and AN conceived and planned the experiments. MG and OC carried out the experiments. SB wrote the manuscript. JR, PV, CD and AN provided critical feedback. AN and SB supervised the project. We thank Anissa Bekhtari, trainee, for her bioinformatic help in designing the primers.

References

[1] Witkin SS, Linhares IM. Why do lactobacilli dominate the human vaginal microbiota? BJOG. 2017 Mar; 124 (4): 606-611.

[2] Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. Front Physiol. 2015 Mar 25; 6: 81.

[3] Chee WJY, Chew SY, Than LTL. Vaginal microbiota and the potential of Lactobacillus derivatives in maintaining vaginal health. *Microb Cell Fact*. 2020; 19 (1): 203. Published 2020 Nov 7.

[4] Eryilmaz, M., Gurpinar, S. S., Palabiyik, I. M., Guriz, H., and Gerceker, D. (2019). Molecular Identification and Antimicrobial Activity of Vaginal Lactobacillus sp. Curr Pharm Biotechnol. 2018; 19 (15): 1241-1247.

[5] Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., et al. (2011). Vaginal microbiome of reproductive-age women. Proc. Natl. Acad. Sci. U.S.A. *108 Suppl 1*, 4680–4687.

[6] Douillard, F. P., and de Vos, W. M. (2014). Functional genomics of lactic acid bacteria: from food to health. Microb. Cell Fact. *13 Suppl 1*, S8.

[7] Chen, X., Lu, Y., Chen, T., & Li, R. (2021). The Female Vaginal Microbiome in Health and Bacterial Vaginosis. *Frontiers in cellular and infection microbiology*, *11*, 631972.

[8] Foxman, B., Muraglia, R., Dietz, J.-P., Sobel, J. D., and Wagner, J. (2013). Prevalence of recurrent vulvovaginal candidiasis in 5 European countries and the United States: results from an internet panel survey. J Low Genit Tract Dis *17*, 340–345.

[9] Boyd Tressler A, Markwei M, Fortin C, Yao M, Procop GW, Soper DE, Goje O. (2021). Risks for Recurrent Vulvovaginal Candidiasis Caused by Non-Albicans Candida Versus Candida Albicans. J Womens Health (Larchmt).

[10] Bradshaw, C. S., Tabrizi, S. N., Fairley, C. K., Morton, A. N., Rudland, E., and Garland, S. M. (2006). The association of Atopobium vaginae and Gardnerella vaginalis with bacterial vaginosis and recurrence after oral metronidazole therapy. J. Infect. Dis. *194*, 828–836.

[11] Ferris, M. J., Masztal, A., Aldridge, K. E., Fortenberry, J. D., Fidel, P. L., and Martin, D. H. (2004). Association of Atopobium vaginae, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. BMC Infect. Dis. *4*, 5.

[12] Polatti, F. (2012). Bacterial vaginosis, Atopobium vaginae and nifuratel. Curr Clin Pharmacol *7*, 36–40 Koelmans, A., Nor, N. H., Hermsen, E., Kooi, M., Mintenig, S., & France, J. D. (2019). Microplastics in freshwaters and drinking water: Critical review and assessment of data quality. Water Research, 155, 410 - 422.

[13] Verstraelen, H. (2008). Cutting edge: the vaginal microflora and bacterial vaginosis. Verh. K. Acad. Geneeskd. Belg. *70*, 147–174.

[14] Nugent, R. P., Krohn, M. A., & Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of Clinical Microbiology*, *29* (2), 297–301.

[15] Mckinnon, L. R., Achilles, S. L., Bradshaw, C. S., Burgener, A., Crucitti, T., Fredricks, D. N., et al. (2019). The Evolving Facets of Bacterial Vaginosis: Implications for HIV Transmission. *AIDS Research and Human Retroviruses*, *35* (3), 219–228.

[16] Ravel, J., Brotman, R. M., Gajer, P., Ma, B., Nandy, M., Fadrosh, D. W., et al. (2013). Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. *Microbiome*, *1* (1), 29.

[17] Koressaar T and Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics 2007; 23 (10): 1289-1291.

[18] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen SG. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012 Aug 1; 40 (15): e115.

[19] Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215: 403-410.

[20] Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., and Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. Transl Res *160*, 267–282.

[21] Onderdonk AB, Delaney ML, Fichorova RN. The Human Microbiome during Bacterial Vaginosis. Clin Microbiol Rev. 2016 Apr; 29 (2): 223-38.

[22] Ling, Z., Kong, J., Liu, F., Zhu, H., Chen, X., Wang, Y., Li, L., Nelson, K. E., Xia, Y., and Xiang, C. (2010). Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. BMC Genomics *11*, 488.

[23] Liu, M.-B., Xu, S.-R., He, Y., Deng, G.-H., Sheng, H.-F., Huang, X.-M., Ouyang, C.-Y., and Zhou, H.-W. (2013). Diverse vaginal microbiomes in reproductive-age women with vulvovaginal candidiasis. PLoS ONE *8*, e79812.