

## Diagnosis of aerobic vaginitis by quantitative real-time PCR

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Received: 11 October 2015 / Accepted: 29 December 2015 / Published online: 18 January 2016  
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### Abstract

**Purpose** To evaluate a real-time PCR-based technique to quantify bacteria associated with aerobic vaginitis (AV) as a potential test.

**Methods** Vaginal samples from 100 women were tested by wet-mount microscopy, gram stain and quantitative real-time PCR targeting *Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Escherichia coli*, *Streptococcus agalactiae*, *S. aureus*; *Lactobacillus* spp. AV diagnosis obtained by wet-mount microscopy was used as reference.

**Results** Some level of AV was diagnosed in 23 (23.7 %) cases. Various concentrations of *Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp. were detected in all patients. *Enterococcus* spp. were detected in 76 (78.3 %) cases. Summarized concentrations of aerobes were tenfold higher in AV-positive compared to AV-negative cases [7.30lg vs 6.06lg ( $p = 0.02$ )]. Concentrations of aerobes in severe, moderate and light AV cases did not vary significantly ( $p = 0.14$ ). Concentration of lactobacilli was 1000-fold lower in AV-positive cases compared to normal cases (5.3lg vs 8.3lg,  $p < 0.0001$ ). *Streptococcus* spp. dominated in the majority of AV-positive cases [19/22

(86.4 %) samples]. The relation of high loads of aerobes to the low numbers of Lactobacilli are a reliable marker for the presence of AV and could substitute microscopy as a test.

**Conclusions** PCR may be a good standardized substitution for AV diagnosis in settings where well-trained microscopists are lacking.

**Keywords** Aerobic vaginitis · Polymerase chain reaction · Flora alterations · Wet-mount microscopy · Bacterial vaginosis · Abnormal vaginal flora · Lactobacilli · Vaginitis

### Introduction

Aerobic vaginitis (AV) is defined as a disruption of the lactobacillary flora, variably accompanied by signs of inflammation and the presence of a rather scarce, predominantly aerobic microflora, composed of enteric commensal pathogens [1]. Although AV is being studied for more than 10 years [2], data about its pathogenesis are limited. It has been reported that AV is registered in 8.3–10.8 % of pregnant women [3, 4] and in 5–23.74 % of women reporting vaginal complaints [5–7]. Major microorganisms associated with this condition are *Escherichia coli*, *Streptococcus* spp. (including group B streptococci), *Staphylococcus aureus*, coagulase-negative staphylococci, *Staphylococcus epidermidis*, *Enterococcus faecalis*, [1, 2, 5, 8]. Although comprehensive data is unavailable, it has been previously demonstrated that AV (or specific bacteria) can be associated with chorioamnionitis, funisitis, neonatal sepsis and preterm birth [3, 9–15]. This association can be explained by significantly elevated levels of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6,

This work was an award winning presentation at the 1st ISIDOG/9th ESIDOG European conference in Riga, 29th Oct–1st Nov 2015.

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IL-8) [2] and sialidase activity [7], which has been previously linked to preterm birth [16, 17]. Still the role of individual aerobic bacteria in AV has not been evaluated thoroughly, nor have molecular biology methods been investigated properly as a potential substitute for microscopy as a diagnostic tool. As molecular techniques can provide comprehensive data about vaginal flora composition, the aim of this study was to investigate vaginal microflora using real-time PCR-based technique in AV-positive and AV-negative patients, and to assess the potential to use this as a diagnostic tool.

## Methods

One hundred Caucasian women undergoing annual routine gynecological examination in the Clinical Diagnostics and Research Center (outpatient department) in May, 2011, were enrolled in this study. All participants were asymptomatic employees of Central Research Institute for Epidemiology, Moscow, Russia. All participants were at least 18 years of age, and not menstruating at the time of enrollment. Each participant signed an informed consent form. Ethical approval for this study was obtained through the Central Research Institute for Epidemiology Ethics Committee (Moscow, Russia). The study also aimed to evaluate a real-time PCR-based kit developed to diagnose bacterial vaginosis. These data were published in a former paper [18].

Three vaginal samples were derived from each participant: one for PCR analysis, one for Gram stain and one for wet-mount microscopy. Gram stain was used for BV diagnosis; these results were published in a separate communication [18]. Vaginal fluid was collected with cotton swabs from the lateral upper vaginal wall using an unlubricated speculum. Information regarding symptoms and medication use during 4 weeks prior to enrollment was collected.

Smears for microscopy were air-dried and sent to Femicare, Tienen, Belgium, for blinded analysis by microscopy. Microscopy of wet smears was performed according to criteria described by Donders et al. [2] resulting in AV score (<3 scores—no signs of AV; 3–4 scores—light AV; 5–6 scores—moderate AV; >6 scores—severe AV). The lactobacillary grades were the basis for a composite score to which any of the four following variables were added: leucocytes [score 0 corresponds to fewer than 10 leucocytes per high power field (HPF, 400× magnification), more than 10 leucocytes per HPF correspond to score 1 if there are fewer than 10 leucocytes per epithelial cell and score 2 if there are more than 10]; presence of toxic leucocytes (the score is 0 if there are no such leucocytes, 1 if <50 % are toxic, and 2 if >50 % of

the leucocytes have a toxic appearance); presence of parabasal cells (no parabasal cells: score = 0; parabasal cells representing <10 % of the epithelial cells: score = 1; parabasal cells representing >10 % of the epithelial cells: score = 2); and background flora [score 0 if the background flora is unremarkable or shows debris and bare nuclei from lysed epithelial cells (cytolysis), score 1 if the lactobacillary morphotypes are very coarse or resemble small bacilli (other than lactobacilli), and 2 if there are prominent cocci, or chained cocci visible] [2].

Also, other findings, like presence of bacterial vaginosis, Candida, sperm or artifacts were noted.

Specimens for real-time PCR were stored in 0.5 ml of buffer-salt solution supplemented with mucolytic, preservative, and stabilizing agents (Transport Medium with Mucolytic Agent, AmpliSens, InterLabService, Russia). DNA extraction was performed using silica-based manual technique “DNA-Sorb-AM” (AmpliSens, InterLabService, Russia), which was shown to have high efficacy of DNA extraction and low proportion of inhibition in previous studies [19]. Primers and probes targeting *Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *E. coli*, *Streptococcus agalactiae*, *Staphylococcus aureus* were designed to detect corresponding species/genus (Table 1). Quantification of *Lactobacillus* spp. and total bacterial count was performed previously for all those samples [18].

TE-buffer was used to obtain desired concentrations of primers and probes. Commercially available DNTPs, Tag-F polymerase and buffer (PCR-mix-2-red) were used (InterLabService, Moscow, Russia). Standard samples made quantitative analysis possible; concentrations were registered as number of genome equivalents (GE) per 1 ml of transport medium.

It was not possible to perform PCR analysis for 1 AV-positive sample due to lacking sample (PCR was performed for 99 remaining samples).

For statistical analysis GraphPad Prism V.6.00 software was used.

## Results

Three samples could not be analyzed as a result of insufficient smear quality on wet-mount microscopy. Mean age of participating women was  $37.6 \pm 10.6$  years. Of the 77 women providing information regarding menstrual cycle day, 39 (50.6 %) were in the follicular phase of their menstrual cycle. Two (2 %) participants were pregnant (12 and 35 weeks of gestation). Although no woman was presenting with symptoms, upon solicitation, 13 (13 %) admitted to have increased vaginal discharge, and 1 (1 %) itching. Antibiotic use during 4 weeks prior to enrollment

**Table 1** Primers and probes used for amplification

Bacteria	Primers, probes	Concentration, pmol/react
<i>Enterobacteriaceae</i>	gCg-gCC-CCC-Tgg-ACg-AAg-A	0.7
<i>Enterobacteriaceae</i>	gCC-TCA-Agg-gCA-CAA-CCT-CCA-A	0.7
<i>Enterobacteriaceae</i>	Cgg-TTC-AAg-ACC-ACA-ACC-TCT-AA	0.7
<i>Enterobacteriaceae</i>	(FAM)CgC-TCA-ggT-gCg-AAA-gCg-Tgg-g(BHQ1)	0.2
<i>Staphylococcus</i> spp.	gCT-ACA-CAC-gTg-CTA-CAA-Tgg-ACA-A	0.7
<i>Staphylococcus</i> spp.	CgT-ATT-CAC-CgT-AgC-ATg-CTg-ATC-TA	0.7
<i>Staphylococcus</i> spp.	(R6G)CAg-CgA-AAC-CgC-gAg-gTC-AAg-C(RTQ)	0.2
<i>Staphylococcus</i> spp.	(R6G)CAg-CTA-AAC-CgC-gAg-gTC-ATg-C(RTQ)	0.25
<i>Streptococcus</i> spp.	gCT-ACA-CAC-gTg-CTA-CAA-Tgg-TT	0.7
<i>Streptococcus</i> spp.	CAg-CCT-ACA-ATC-CgA-ACC-gAg-ATT	0.6
<i>Streptococcus</i> spp.	CAg-CCT-ACA-ATC-CgA-ACC-gAg-ACT	0.6
<i>Streptococcus</i> spp.	(ROX)CgC-AAg-CCg-gTg-ACg-gCA-AgC(RTQ)	0.2

was reported by 7 (7 %) of participants, oral probiotics by 2 (2 %), antiseptics by 1 (1 %). Oral contraceptives were used by 7 (7 %), and spermicidal cream by 1 (1 %) of the women, others reported condom use and/or coitus interruptus.

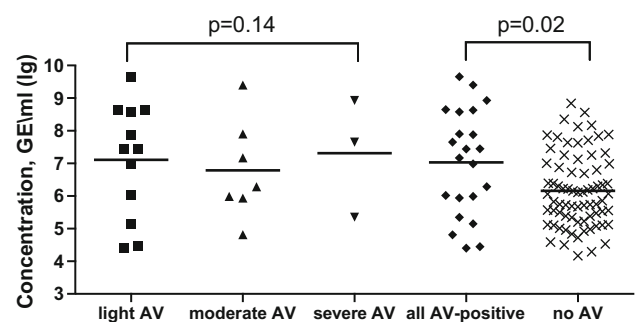
AV was diagnosed in 23 (23.7 %) cases. Light AV was revealed in 13 (13.4 %), moderate in 7 (7.2 %), and severe in 3 (3.1 %) cases.

Various concentrations of *Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp. were detected in all patients. *Enterococcus* spp. were detected in 76 (78.3 %) cases [20/23 AV-positive cases and 56/74 AV-negative cases ( $p = 0.198$ )]. Summarized median concentrations of aerobes detected [ $\lg^1$  (GE/ml)] were as follows: 7.44 for light AV, 6.28 for moderate AV and 7.65 for severe AV cases ( $p = 0.14$ ); median concentration of aerobes for all AV-positive cases was 7.30lg vs 6.06lg in AV-negative women ( $p = 0.02$ ) (Fig. 1).

Median concentrations of *Enterobacteriaceae* in AV-positive and AV-negative groups were  $2.7 \times 10^3$  GE/ml and  $1.5 \times 10^3$  GE/ml, respectively ( $p = 0.38$ ); *Staphylococcus* spp.  $5.7 \times 10^4$  GE/ml and  $7.8 \times 10^4$  GE/ml, respectively ( $p = 0.30$ ); *Streptococcus* spp.  $1.2 \times 10^7$  GE/ml and  $8 \times 10^5$  GE/ml, respectively ( $p = 0.03$ ); *Enterococcus* spp.  $3.5 \times 10^3$  GE/ml and  $3.3 \times 10^2$  GE/ml, respectively ( $p = 0.003$ ) (Fig. 2).

Among four groups of aerobic bacteria *Enterobacteriaceae* dominated in 1/22 (4.5 %) of AV-positive samples, *Staphylococcus* spp.—in 2/22 (9.1 %) samples, *Streptococcus* spp.—in 19/22 (86.4 %) samples, *Enterococcus* spp. in—0/22 samples (Fig. 3). All severe ( $N = 3$ ) and moderate ( $N = 7$ ) AV cases were associated with *Streptococcus* spp., while three samples were dominated by *Enterobacteriaceae* and *Staphylococcus* spp. (light AV on microscopy).

<sup>1</sup> Logarithm to the base ten.

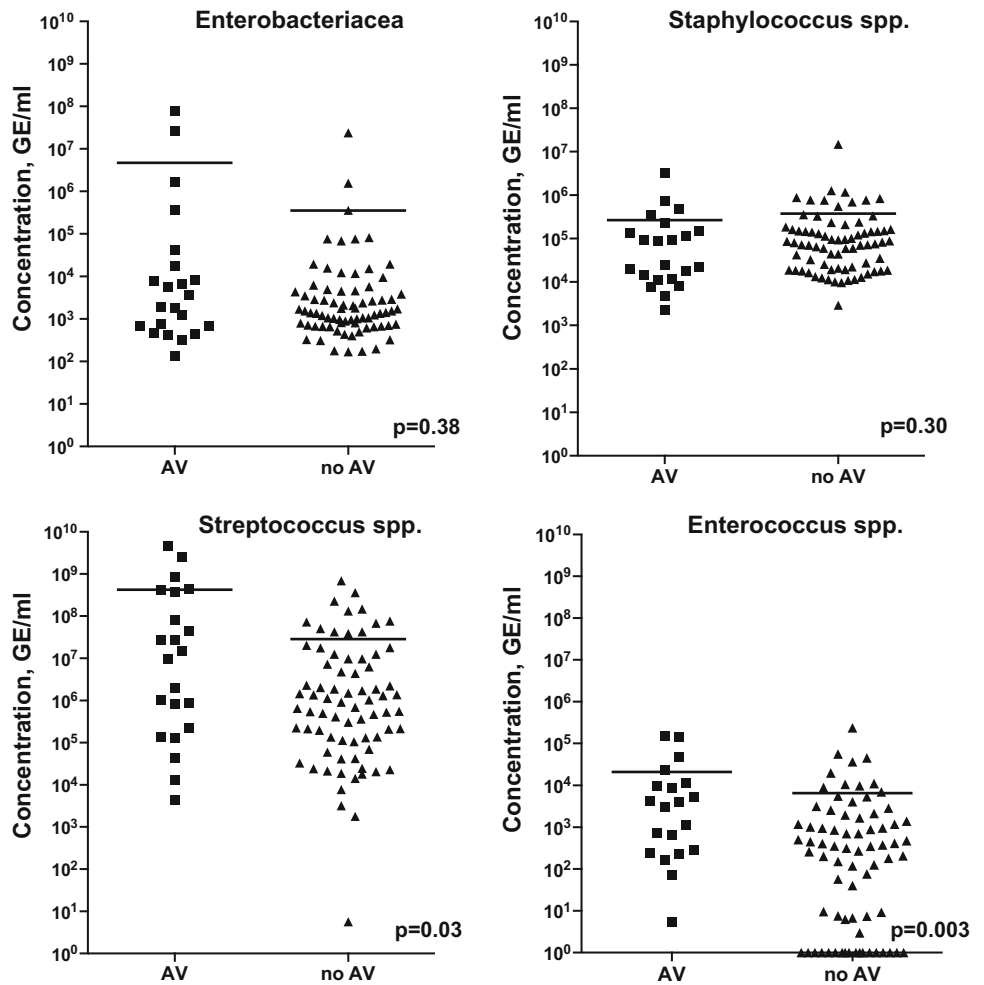


**Fig. 1** Summarized concentrations of aerobes (*Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp.) detected by real-time PCR are displayed for five groups of patients: (1) patients with light AV (light AV on the graph), (2) moderate and (3) severe AV (detected by wet-mount microscopy); (4) all patients with AV (all AV-positive) and (5) patients without AV by microscopy. Horizontal bars reflect median concentrations of aerobes in each group

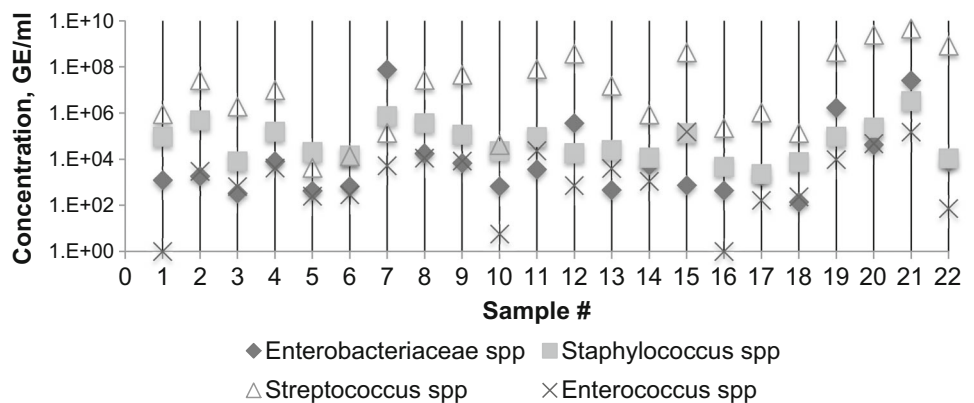
In the one *Enterobacteriaceae*—dominated AV sample, *E. coli* was detected as the sole microorganism with a concentration of *Enterobacteriaceae* being equal to that of *E. coli* ( $10^7$  GE/ml). In the 2 *Staphylococcus* spp.—dominated cases *S. aureus* was not detected. *S. agalactiae* was detected in all 19 (100 %) *Streptococcus* spp.—dominated AV cases. Of note, in 10 samples, the concentration of *S. agalactiae* was >11 g lower than the concentration of *Streptococcus* spp.

In 14 of the studied samples we demonstrated that *Lactobacillus* spp. concentration was very low, while at the same time *G. vaginalis* and *A. vaginae* concentration was much less than the total bacteria concentration. According to the formula ( $\lg[\text{Bact}] - \lg[\text{Lacto}] > 1$  and  $\lg[\text{Bact}] - \lg[\text{Gv} + \text{Av}] > 2$ ) [18], this resulted in the conclusion “Flora alteration other than BV” (lactobacillary flora substituted by bacteria other than *G. vaginalis* and/or *A. vaginae*), which according to the current data coincides with the diagnosis of AV. In the current study it was indeed demonstrated by microscopy that those 14 samples were all

**Fig. 2** Concentrations of various aerobes detected by real-time PCR in AV-positive and AV-negative patients (by wet-mount microscopy) are displayed. Four graphs correspond to four major groups of microorganisms studied: *Enterobacteriaceae* (top left), *Staphylococcus* spp. (top right), *Streptococcus* spp. (bottom left), *Enterococcus* spp. (bottom right). Horizontal bars reflect median concentrations of aerobes in each group



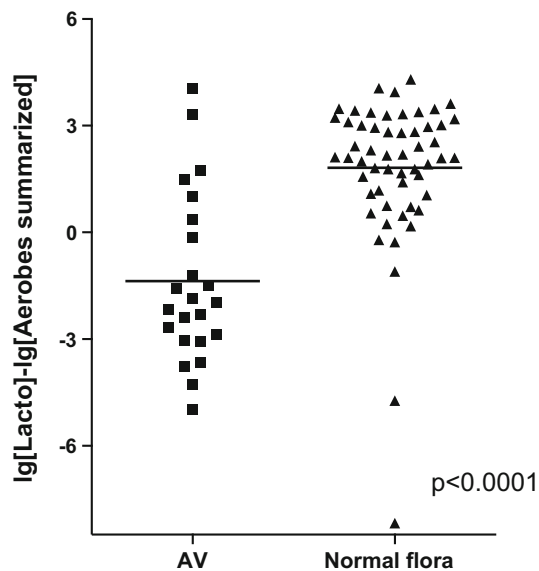
**Fig. 3** Concentrations of *Enterobacteriaceae* (filled diamonds), *Staphylococcus* spp. (filled squares), *Streptococcus* spp. (triangles), *Enterococcus* spp. (cross marks), detected by real-time PCR in each AV-positive case (by wet-mount microscopy) are displayed (totally 22 positive samples)



AV-positive (PCR demonstrated that aerobes substituted lactoflora in all samples), while other nine AV-positive samples were mixed conditions (AV+bacterial vaginosis).

As in all those samples concentrations of *Lactobacilli* were decreased (compared to the total bacterial count), we calculated the difference between concentrations of *Lactobacilli* and aerobes detected in AV-positive samples (by

microscopy) and samples with no flora alteration ( $\lg[\text{Lacto}] - \lg[\text{Aerobes summarized}]$ ) (Fig. 4). It was clearly demonstrated that in AV-positive cases aerobes prevail (median difference  $-1.98$ , in other words, concentration of aerobes dominate over concentration of *Lactobacilli* by 2 logs), compared to samples with normal flora, where *lactobacilli* dominate (median difference  $2.12$ ,  $p < 0.0001$ ).



**Fig. 4** Difference between concentrations of Lactobacilli and aerobes (by real-time PCR) detected in AV-positive (AV on the graph) samples and samples with no flora alteration (normal flora on the graph) by wet-mount microscopy are displayed. *Y-axis* reflects the difference between logarithms of concentrations of Lactobacilli and all aerobes detected in the sample ( $\lg[\text{Lacto}] - \lg[\text{Aerobes summarized}]$ ). *Horizontal bars* reflect median concentrations of aerobes in each group

## Discussion

The aim of the study was to investigate vaginal microflora using real-time PCR-based technique in AV-positive and AV-negative patients in order to find an alternative, PCR-based technique to diagnose AV. Samples from 100 asymptomatic women undergoing routine annual examination were evaluated by wet-mount microscopy and real-time PCR-based techniques. Surprisingly, some level of AV was detected by microscopy in 23.7 % of healthy women. In previous studies such a high prevalence was demonstrated only for women complaining of vaginal discharge [5]. At the same time, severe AV was diagnosed only in 3.1 % of our volunteering subjects, in all other cases only light or moderate AV was demonstrated. It might be subject for future studies if this prevalence is typical for Russian women. Also, if ever molecular biology techniques were to replace microscopy as a diagnostic tool, the potential risk of over-diagnosis has to be monitored closely.

In this study, we found a clear combination of low lactobacilli content and a 10 fold increased number of aerobes in AV, but at the same time we were not successful in finding a threshold for AV severity based on quantification of single aerobic bacteria by real-time PCR.

There are some major limitations of this study: (1) with 100 participants small numbers of particular cases were retained in each subgroup, (2) in some cases not all

possible species of aerobic bacteria could be targeted and (3) normalized concentrations of bacteria versus  $\beta$  globulin were not assessed in this study.

For *Streptococcus* spp. significant diversity was demonstrated, preventing finding a straightforward threshold for reliable AV diagnosis. Therefore simple quantification of aerobic bacteria by real-time PCR is not sufficient for AV detection. Normalized concentrations of these bacteria (using B-globin gene for normalization, for example) may be a better predictor of AV, but this issue was beyond the scope of this study.

Not only *Enterobacteriaceae*, *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. were targeted and quantified, but also the most typical representatives of those groups: *E. coli*, *S. aureus*, *S. agalactiae*, respectively, were typed. There were no AV-positive samples dominated by *Enterococcus* spp, meaning this organism is not a major marker for AV. These results do not support previous findings demonstrating the potential role of *Enterococcus faecalis* as obtained by culture in AV [1].

*E. coli* clearly dominated in one AV sample dominated by *Enterobacteriaceae*, which confirms that *E. coli* can play an important role in some types of AV [1, 2, 8, 15]. *S. aureus* was not detected in 2 *Staphylococcus* spp.—dominated AV cases. Unfortunately coagulase-negative Staphylococci and *Staphylococcus epidermidis* were not quantified in this study; nevertheless results obtained are in a good agreement with existing data regarding the role of different Staphylococci in AV development [1, 2, 8]. Most strikingly, *Streptococcus* spp. dominated in the majority of AV cases (86.4 %) and in all severe and moderate cases. In about half (9/19) of those cases *S. agalactiae* was found as the dominating microorganism, which clearly shows that besides *S. agalactiae*, other streptococci should also be considered as organisms involved in the pathogenesis of AV [1, 2, 8].

The absence of a clear bacterial threshold or the failure to detect a single microbial agent to diagnose AV does not come as a surprise. Indeed, the diagnosis of AV is not only based on the presence of aerobic bacteria alone, but rather on the combined finding of these aerobic bacteria with variable inflammation, epithelial cell immaturity (parabasal cells) and the disruption of the lactobacillary flora [2]. Indeed, if with PCR the absence of anaerobic bacteria in the presence of a lactobacillary disruption is considered, as suggested before [18], this test appears to provide an excellent prediction of the presence of AV. As *Lactobacillus* spp. concentration as well as total bacteria count using real-time PCR were also evaluated in this study, it was clearly demonstrated that in all AV-positive cases *Lactobacillus* spp. were substituted by aerobic bacteria, which is in a good concordance with the very idea of AV being a flora alteration resulting in the decrease of

*Lactobacillus* spp. concentration [2]. As a result we could demonstrate that a decrease of *Lactobacillus* spp. concentration accompanied by high loads of *Enterobacteriaceae*, *Staphylococcus* spp. or *Streptococcus* spp. may be predictive of AV. At the same time diagnosis “vaginitis” itself implies signs of inflammation, which will never allow to achieve a full diagnosis of AV based on PCR results only. Previously we reported that quantitative PCR technique (multiplex real-time PCR using primers and probes targeting *G. vaginalis*, *A. vaginae*, *Lactobacillus* species and total quantity of bacterial DNA) enabled accurate prediction of BV, diagnosed either by wet-mount microscopy or by Nugent score [18].

In settings where well-trained microscopists who can perform accurate assessment of wet-mount or Gram stained smears are lacking, PCR reliably substitutes for AV diagnosis in the presence of clinical signs of inflammation. If in such cases increased concentrations of *Enterobacteriaceae*, *Staphylococcus* spp, or *Streptococcus* spp. and decreased *Lactobacillus* spp. are present, the diagnosis of AV is very likely. Asymptomatic women positive for *Enterobacteriaceae*, *Staphylococcus* spp, or *Streptococcus* spp. by PCR are not AV-positive and do not need any treatment.

#### Compliance with ethical standards

**Funding** This study was funded by Central Research Institute for Epidemiology, Moscow, Russia, and Femicare, Tienen, Belgium.

**Conflict of interest** Authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration.

**Informed consent** Written informed consent was obtained from all individual participants included in the study.

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