Strong correspondence in bacterial loads between the vagina and rectum of pregnant women

Nabil Abdullah El Aila, Inge Tency, Bart Saerens, Ellen De Backer, Piet Cools, Guido Lopes dos Santos Santiago, Hans Verstraelen, Rita Verhelst, Marleen Temmerman, Mario Vaneechoutte

Laboratory of Bacteriology Research, Department of Clinical Chemistry, Microbiology & Immunology, Ghent University, 3Bloka, 185 De Pintelaan, 9000 Ghent, Flanders, Belgium

Department of Obstetrics & Gynaecology, Ghent University Hospital, Ghent University, 185 De Pintelaan, 9000 Ghent, Flanders, Belgium

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Abstract

We sampled the vagina and rectum in 71 pregnant women and bacterial loads of Lactobacillus crispatus, L. jensenii, L. gasseri, L. iners, Gardnerella vaginalis and Atopobium vaginae were determined by culture and quantitative PCR (qPCR).

Culture and qPCR results differed substantially with regard to the evaluation of vaginal and rectal occurrence of the six species tested. The vaginal—rectal prevalence of L. crispatus, L. jensenii, L. gasseri, L. iners, G. vaginalis and A. vaginae as established by culture vs. PCR was 32.3 vs. 91.5%, 32.3 vs. 77.4%, 28.1 vs. 91.5%, 12.6 vs. 68.5%, 12.6 vs. 74.6% and 5.6 vs. 69.0%, respectively.

Using qPCR, a significant positive correlation was found between vaginal and rectal loads of L. crispatus ($p < 0.0001$), L. jensenii ($p < 0.0001$), L. gasseri ($p = 0.005$), L. iners ($p = 0.003$) and A. vaginae ($p = 0.002$).

In summary, significant correlations between quantities of vaginal and rectal lactobacilli and of Atopobium vaginae were established by means of qPCR, indicating strong correspondence of vaginal and rectal microflora, not only in the occurrence of certain species in both niches, but also of cell densities per bacterial species.

Keywords: Atopobium vaginae; Gardnerella vaginalis; Lactobacilli; Pregnant women; qPCR

1. Introduction

The vaginal microflora plays an active role in preventing genital infection (Arouetcheva et al., 2001; Hillier et al., 1993; Tomas et al., 2004). This protective function, i.e., colonization resistance, is primarily related to the presence of bacteria belonging to the genus Lactobacillus, preventing overgrowth of pathogenic microorganisms (Ronqvist et al., 2006). Four species of lactobacilli are now considered predominantly linked to the vaginal microflora: Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners and Lactobacillus jensenii, with L. iners only recently recognized; indeed, it was long overlooked because it stains Gram-negative and does not grow on de Mann Rogosa Sharpe (MRS) agar, the medium typically used to culture lactobacilli (De Backer et al., 2007). In bacterial vaginosis (BV), the predominant lactobacilli are replaced by Gardnerella vaginalis and anaerobic bacteria (Hillier et al., 1993; Eschenbach et al., 1989; Hill, 1993). Recently, Atopobium vaginae has been strongly associated with BV independently by different groups (Burton et al., 2004; Ferris et al., 2004; Fredricks et al., 2005; Romanik...
et al., 2006; Verhelst et al., 2004). Several bacterial species are known to colonize both the gastrointestinal and reproductive tracts, and the rectum has been suggested to play an important role as a source or reservoir of organisms that colonize the vagina (Antonio et al., 1999, 2005; Hallen et al., 1988; Holst, 1990; El Aila et al., 2009a,b). Recently, we showed that the rectum and vagina harbor not only the same bacterial species in 36% of 132 pregnant women studied, but also the same genotypes for 34 (68%) of the 50 species pairs that were available for genotyping (El Aila et al., 2009b). Another indication of rectal colonization of the vagina comes from studies whereby ingestion of probiotics for treatment and prevention of vaginal infections has shown that vaginal colonization can be obtained with these lactobacilli within days after oral application (Reid et al., 2001).

In this study, we used culture and qPCR to detect and quantify four Lactobacillus species, G. vaginalis and A. vaginae in the vagina and rectum of pregnant women.

2. Materials and methods

2.1. Patients

The study was approved by the research ethics committee (IRB protocol nr 2007/096) of Ghent University Hospital, Belgium. Participating women gave written informed consent. Between April and December 2007, paired vaginal and rectal swabs were collected from 71 pregnant women at 35–37 weeks of gestation.

2.2. Sampling procedures

All specimens were collected using nylon flocked swabs submerged in 1 ml of liquid Amies transport medium (eSwab, Copan Diagnostics, Brescia, Italy). For rectal specimens, a swab was carefully inserted approximately 1.5–2 cm beyond the anal sphincter and then rotated to touch the anal crypts. Vaginal samples were obtained through blind swabbing by which the swab was rolled around 360° against the vaginal wall at the midportion of the vault. All study samples were collected by midwives and transported to the Laboratory for Bacteriology Research of the University of Ghent, inoculated on culture media and submerged in DNA extraction buffer within 4 h.

2.3. Culture and Gram staining

A total of 70 μl from Amies liquid transport medium of each of the vaginal and rectal swabs was inoculated onto Columbia CNA agar with 5% sheep blood (Columbia CNA agar, Becton Dickinson, Erembodegem, Belgium) and MRS agar (Oxoid, Hampshire, UK) and incubated at 37 °C in an anaerobic chamber (10% H2, 10% CO2, 80% N2) (BugBox, LedTechno, Heusden-Zolder, Belgium) for 72 h. Another 50 μl of the vaginal swab suspension was taken for Gram stain smear preparation.

Gram stain-based grading was carried out according to Verhelst et al. (2005).

2.4. DNA extraction from samples

DNA extraction from samples was performed using the NucliSENS EasyMAG platform according to the manufacturer’s instructions. Briefly, 200 μl of Amies transport medium from each vaginal and rectal swab was added to easyMAG lysis buffer and incubated for 10 min at room temperature and stored at −80 °C until extraction. Total nucleic acid extraction of the samples was carried out and nucleic acids were eluted in 100 μL of elution buffer.

2.5. DNA extraction from isolates

DNA was extracted from cultured isolates by alkaline lysis as described previously (El Aila et al., 2009b).

2.6. Identification of isolates by tRNA intergenic length polymorphism analysis (tDNA-PCR)

From 71 women, 8 colonies per subject, i.e., one colony of each of the two most abundant colony types from both Columbia CNA and MRS agar plates and for both rectal and vaginal swabs were picked, i.e., a total of 568 isolates, 284 vaginal and 284 rectal. Isolates were identified by means of tRNA intergenic length polymorphism analysis (tDNA-PCR) as described previously (Baele et al., 2000, 2001). Isolates for which no clear-cut identification was obtained by tDNA-PCR were identified by 16S rRNA gene sequencing.

2.7. qPCR

qPCRs for the six target species were carried out as described previously (De Backer et al., 2007).

2.8. Statistical methods

Data were analyzed using the Chi-square test, the Mann-Whitney U-test for two groups and the Kruskal Wallis test for multiple groups. Correlations between the different species were determined by the Spearman (rank) test and reported as Spearman’s rho value (r). All analyses were performed using Graphpad Prism software (http://www.graphpad.com).

3. Results

3.1. Culture results

Table 1, Supplementary Table 1 and Fig. 1 reveal the occurrence of four Lactobacillus species and G. vaginalis and A. vaginae vaginally, rectally, and both vaginally and rectally, as determined by culture and qPCR.

Based on culture data, all six species were recovered more frequently from the vagina (ranging from 4.2% for A. vaginae to 30.9% for L. crispatus and L. jensenii) than from the rectum.
ranging from 1.4% for *A. vaginae* to 11.2% for *L. crispatus* and *L. jensenii*. For most women in whom a species could be cultured rectally, it could also be cultured from the vagina. *A. vaginae* was cultured least frequently, i.e., rectally from only 1.4% of the women and vaginally from only 4.2% of the women. The species most abundantly present overall, according to culture were *L. crispatus*, *L. jensenii* and *L. gasseri*, i.e., in approximately 30% of the women.

### 3.2. qPCR results

qPCR yielded a much higher number of rectally- and/or vaginally-positive women for all six species compared to culture and, in contrast to culture-based results, the number of women who were rectally positive was higher than those who were vaginally positive, except for *L. gasseri* and *L. iners*. The overall prevalence of the six species as established by culture ranged from 5.6 to 32.3% vs. qPCR results, ranging from 69.0% to 91.5%, detailed for each species in Table 1.

The higher number of qPCR-positive women was also reflected in the number of women simultaneously carrying the same species both vaginally and rectally. According to qPCR, those percentages ranged from 30.9% for *A. vaginae* to 60.0% for *L. crispatus*, whereas, according to culture, the percentage of women positive for both vagina and rectum ranged from 0% for *A. vaginae*, *G. vaginalis* and *L. iners* to 9.8% for *L. crispatus* and *L. jensenii*.

Most importantly, when using qPCR, significant positive correlations were found between vaginal and rectal colonization, not only with respect to the presence of the same species rectally and vaginally, but also with respect to the bacterial load at both sites, i.e., there was a significant degree of correspondence between vaginal and rectal bacterial loads for five of the six species: *L. crispatus* (r = 0.80, p < 0.0001, Fig. 2A), *L. gasseri* (r = 0.45, p = 0.005, Fig. 2C), *L. iners* (r = 0.51, p = 0.003, Fig. 2D), *L. jensenii* (r = 0.85, p < 0.0001, Fig. 2B) and *A. vaginae* (r = 0.60, p = 0.002, Fig. 2F). Only for *G. vaginalis* were we unable to establish a significant correlation between vaginal and rectal bacterial load (r = 0.23, p = 0.172), although Fig. 2E indicates that, for *G. vaginalis* as well, vaginal loads largely corresponded to rectal loads for most women.

### 3.3. Culture vs. qPCR results

Panels a–f in Fig. 2 demonstrate that culture-positive women mainly corresponded to those in whom a high bacterial load was established using qPCR. Women who were culture-positive, irrespective of whether it was only from the vagina, only from the rectum or from both vagina and rectum,
in general had high qPCR bacterial loads in both vagina and rectum. Correspondingly, few culture-negative women had high bacterial loads according to qPCR. This is especially clear for *L. crispatus* and *L. jensenii*, for which most culture-positive women were found.

When using culture, all species were recovered mostly only from the vagina, whereas when using qPCR, most species were recovered from both vagina and rectum except for *A. vaginae*, which was recovered mostly from the rectum (Fig. 1).

### 3.4. Presence and load of different bacterial species in the vagina according to category of vaginal microflora

In this study, we largely confirmed our previous findings (De Backer et al., 2007). Briefly, *L. crispatus* was present in the vagina in significantly higher numbers in grade Ia and Iab samples (median: 7 log 10 cells/ml) than in non-grade I samples. The loads of *G. vaginalis* and *A. vaginae* in grade I were significantly lower than those in grades II and III, but did not
differ significantly between grades II and III. Both species were present, respectively, in 10 and 7 of the 12 grade II samples. All six grade III samples were positive for *G. vaginalis*, and four of these were also positive for *A. vaginae*. Forty-two vaginal samples (59%) were positive for *G. vaginalis* by qPCR, while 18 of them, i.e., 43%, were also positive for *A. vaginae*.

Overall, qPCR established significant positive correlations between bacterial loads in the different grades for *G. vaginalis* and *A. vaginae*, both vaginally \(r = 0.396, p = 0.0006\) and rectally \(r = 0.40, p = 0.0001\). Negative correlations were found between vaginal loads of *L. crispatus* and *G. vaginalis* \(r = -0.363, p = 0.001\) and between those of *L. crispatus* and *A. vaginae* \(r = -0.261, p < 0.05\).

We also compared rectal vs. vaginal bacterial load per class of VMF, separated into 3 classes as grades Ia and Iab (normal VMF), Ib and I-like (‘intermediate’ VMF) and II and III (disturbed VMF), whereby differences between the loads of vagina and rectum equal to or more than 0.9 log were considered different, and differences lower than log 0.9 were considered identical. In Supplementary Table 1, loads that were higher compared to the opposite niche per subject are underlined. Table 2 summarizes these data.

The 27 women with intermediate class VMF had comparable bacterial loads for most species between the two niches. For most women, vaginal loads of *L. gasseri* were equal to or higher than rectal loads. Among a total of 71 women, only in four was the rectal load of *L. gasseri* higher than the vaginal load.

For *L. iners*, most women had comparable vaginal and rectal loads independently of the class of VMF, except for 15 out of 27 women with intermediate VMF, for whom *L. iners* was present in higher loads in the vagina (compared to only one woman with higher rectal load). For the 26 women with normal VMF, the vaginal load of *L. crispatus* was higher than that of the rectal load in 21 cases \(P = 0.0002\) and that of *L. jensenii* was higher in 11 cases \(P < 0.05\), with only 2 women in whom the rectal load was highest (for both species), whereas there were more women with a lower vaginal load of *G. vaginalis* and *A. vaginae* compared to the rectal load.

In contrast, for the 18 women with disturbed VMF, the vaginal load of *G. vaginalis* and *A. vaginae* was higher than the rectal load in 12 and 6 women, respectively, with only two women having a higher rectal load of *G. vaginalis* and one woman with a higher rectal load of *A. vaginae* \(P < 0.05\); the vaginal load of *L. crispatus* was lower than the rectal load in 7 women, with only 2 women having a higher rectal load of this species.

### 4. Discussion

In this study, we determined not only the presence but also the quantity of six bacterial species in both the vagina and the rectum, sampled simultaneously for each of 71 pregnant women. This was done by both culture and qPCR.

Our findings are in agreement with previous studies indicating that grade III vaginal microflora (VMF) is associated with a significant increase in *G. vaginalis* and *A. vaginae* and with a drop in (most) *Lactobacillus* species, and that qPCR may assist in the diagnosis of grade III VMF, indicative of BV, when clinical symptoms such as malodorous vaginal discharge are present.

Bradshaw et al. (2006) used qPCR for *A. vaginae* for a follow-up study of recurrent BV before and after treatment with oral metronidazole. Ferris et al. (2007) applied qPCR for *A. vaginae* to samples of six BV patients before and after treatment with topical metronidazole gel. Fredricks et al. (2009) described changes in vaginal bacterial concentrations following metronidazole therapy for BV and indicated that quantification of particular vaginal bacteria by PCR may be useful for monitoring the response to antibiotic therapy. Zarfard et al. (2002) and Sha et al. (2005a, b) quantified lactobacilli in cervico-vaginal lavage samples using a qPCR format, which picked up only *L. crispatus* and *L. jensenii*, and found a decline in the number of lactobacilli in BV. De Backer et al. (2007) performed a study on 71 vaginal samples with the same primer sets as used here and showed a decline in *L. crispatus*, *L. jensenii* and *L. gasseri* and an increase in *L. iners*, *G. vaginalis* and *A. vaginae* in most grade III samples. Menard et al. (2008) reported that qPCR can be used to objectively diagnose BV at well-defined cut-off values for concentrations of *A. vaginae* and *G. vaginalis*. Biagi et al. (2009) showed that bacterial vaginosis is associated with a significant increase in *Prevotella*, *Atopobium*, *Veillonella* and *G. vaginalis* and a decrease in *Lactobacillus* species, and with strong variability over time and between individuals, whereas the vaginal microflora under normal conditions is homogeneous and stable over time.
However, to our knowledge, qPCR has not yet been used to compare in a direct and quantitative manner the rectal and vaginal microflora of women, as was done in this study, and which could throw light on the origin of bacteria associated with grade III VMF and BV.

A first striking finding was that, when compared to culture, a high number of pregnant women are positive for most species studied by qPCR. This might be due to the high sensitivity of PCR, but also to the low specificity of this approach. However, the latter explanation is contradicted by the finding that women who were culture-positive generally also had the highest bacterial loads according to qPCR. In addition, the fact that high vaginal load in qPCR usually corresponded to high rectal load is an argument for explaining these findings as resulting from the high sensitivity of qPCR rather than from its possible low specificity. In summary, qPCR is much more sensitive than culture for determining the vaginal and rectal presence of bacteria, and qPCR indicates a much higher correspondence between vaginal and rectal bacterial loads than can be established by culture. It should be noted that we picked a total of 8 colonies for each woman, whereas in routine culture applications only one or a few colonies are selected for further characterization, i.e., the sensitivity of culture in routine laboratories can be considered as even lower than in this study. This is not unexpected: *A. vaginae*, *G. vaginalis* and *L. iners* are fastidious species which can be easily overlooked or overgrown when dealing with complex intestinal microflora. In fact, the rectal presence of all species was underestimated by culture, as shown in Fig. 1.

The finding that vaginal and/or rectal samples which are culture-positive for certain bacterial species contain high loads of that species, as quantified by qPCR, indicates that culture generally detects the *Lactobacillus* species *G. vaginalis* and *A. vaginae* only when these species are present at higher concentrations.

Using qPCR, we established a mean vaginal bacterial load of 109.1 cells/ml for *G. vaginalis* in grade III, which corresponds well to the value of 109.2 cells/ml that we reported previously (De Backer et al., 2007) and also to the value of 109.6 cells/ml reported by Zariffard et al. (2002). In this study, we confirm the coexistence of *A. vaginae* and *G. vaginalis*, which was recently documented in 78–96% of vaginal samples from women with BV, whereas this association was present in only 5–10% of samples from women with normal vaginal microflora (Bradshaw et al., 2006; Verhelst et al., 2005). In agreement with these findings, Menard et al. (2008) reported that qPCR-based quantification of *A. vaginae* (≥10⁸ copies/mL) and *G. vaginalis* (≥10⁹ copies/mL) had the highest predictive value for diagnosis of BV.

Most importantly, by means of qPCR, we found a high degree of correspondence between vaginal bacterial load and rectal bacterial load for five of the six species, i.e., *A. vaginae*, *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*. Only for *G. vaginalis* were we unable to establish a significant correlation between vaginal and rectal bacterial load, although, for most women, a higher vaginal load corresponded to a higher rectal load for this species as well. We confirmed that the high rectal loads of *A. vaginae* and *G. vaginalis* were not due to aspecific amplification (e.g., of bifidobacteria in the case of the *G. vaginalis* qPCR) by 16S rRNA gene sequencing of five qPCR products positive for *A. vaginae* and four qPCR products positive for *G. vaginalis*. This showed that the sequences of these qPCR products were indeed identical to reference strains of both species (data not shown).

This quantitative approach reinforces qualitative results of several previous studies, which indicated the rectum as the reservoir for vaginal microflora. For example, the rectum seems to be the primary habitat of *Candida* spp. in patients with candidal vulvovaginitis (Miles et al., 1977). Meyn et al. (2009) showed that rectal colonization with group B streptococci (GBS) was the most significant predictor of vaginal colonization, suggesting that the vagina becomes colonized with GBS as a result of transfer of the organism from the rectum to the vagina. This is not in contradiction with our previous genotyping study, which showed a high correspondence of vaginal and rectal colonization with GBS, also at the strain level (El Aila et al., 2009a). Hallen et al. (1988) reported simultaneous occurrence of *Mobiluncus* spp. in the vagina and rectum. Holst (1990) reported that reservoirs of *Mobiluncus* spp., *G. vaginalis* and *Mycoplasma hominis* are in the intestinal tract. We previously showed a high correlation between vaginal and rectal microflora, not only with regard to species composition but also with regard to the presence of genotypically identical strains (El Aila et al., 2009a,b). To our knowledge, the present study is the first that also indicates a clear quantitative correspondence between bacterial loads present in the vagina and rectum, for at least five of the six species studied.

Although recurrent BV might be explained by the existence of ineradicable biofilms, shown previously to be formed by *G. vaginalis* and *A. vaginae* during BV (Swidsinski et al., 2005), another explanation may be recolonization from the rectum, whereby some women may be more prone to recolonization with BV-associated organisms because of the presence of high loads of these bacteria in their rectum. The proneness of certain women to colonization with group B streptococci or uropathogenic *Escherichia coli* might be explained in a similar manner, but requires further investigation.

The data whereby vaginal and bacterial loads are compared per subject according to class of VMF (Table 2) indicate that, despite the simultaneous presence in both vagina and rectum of most species studied and despite the overall congruence in bacterial loads between vagina and rectum for most subjects, there are clear differences between women with normal VMF and those with disturbed VMF. The species associated with normal VMF (*L. crispatus* and *L. jensenii*) are more abundant vaginally in women with normal VMF, whereas the species associated with disturbed VMF, i.e., *G. vaginalis* and *A. vaginae*, are present in higher loads in the vagina compared to the rectum in women with disturbed VMF.

Finally, the prevalence and role of *L. iners* as part of the vaginal microflora remains intriguing. In this study, using qPCR, *L. iners* was detected vaginally and rectally in high
numbers when compared to culture and when compared to other studies of the rectal microflora that were culture-based (Antonio et al., 1999, Marrazzo et al., 2009). In our study, the prevalence of L. iners in the rectum was 2.8% using culture vs. 52.0% using qPCR. In previous studies (Antonio et al., 2005; Marrazzo et al., 2009), L. iners was not isolated from the rectum alone; here, however, using qPCR, we could detect it from the rectum only in 9.8%, and from both the vagina and the rectum in 42.2%, of subjects.

Establishing the prevalence of L. iners in the vagina has thus far been cumbersome when relying on culture and Gram stain, because L. iners cannot be propagated on conventional Lactobacillus-selective media such as Rogosa or MRS agar (Falsen et al., 1999) and because the cell morphology of L. iners is Gram-negative cocccobacillus rather than Gram-positive bacillus, as for regular lactobacilli (De Backer et al., 2007).

Since it is the predominant Lactobacillus species in BV microflora (De Backer et al., 2007; this study), but goes largely undetected by culture and on Gram stain, this has led to the false assumption that BV microflora is devoid of lactobacilli. Moreover, because also the Gram-positive G. vaginalis stains Gram-negative (De Backer et al., 2007), this probably explains the description of BV microflora as dominated by ‘anaerobic Gram-negatives’, while two species, usually present in high numbers in BV samples, i.e., G. vaginalis and L. iners, are Gram-positives.

The principle of using exogenous human bacteria to restore ‘normal’ microflora has been applied to the gut, vagina, and other organs. This was recently fully reviewed (Lenoir-Wijnkoop et al., 2007, Barrons and Tassone, 2008). Barrons and Tassone (2008) identified three studies that reported a reduction in recurrence of BV associated with the use of oral probiotics and four that did not. Probiotics could provide a natural, safe and effective means of stabilizing fluctuating vaginal microflora and thereby lower the risk of infection in healthy women as well as those prone to urogenital disease.

Our results confirm that vaginal probiotics can easily be administered orally, thereby colonizing the vagina within several days. Daily oral intake of probiotic strains Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 resulted in asymptomatic BV patients reverting to normal lactobacilli-dominated vaginal microflora (Reid et al., 2001, 2003).

Direct vaginal application of probiotics may be preferable for rapid treatment of BV. However, the ability to deliver vaginal probiotics orally could be more convenient for long-term prevention (MacPhee et al., 2010).

In conclusion, we established significant differences in detection of six bacterial species of the vaginal and rectal microflora using culture and qPCR. The carriage rate was 3-fold (for L. crispatus) to 17-fold (for A. vaginae) higher according to qPCR than according to culture despite the fact that, for each subject 8 colonies were picked and identified. Women were positive for the six species in 4.0–32.3% of cases according to culture, compared to 68.5–91.5% of cases according to qPCR. In addition, and most importantly, using qPCR, significant correlations between quantities of vaginal and rectal lactobacilli and of A. vaginae were established, indicating strong correspondence of vaginal and rectal microflora not only with regard to simultaneous occurrence of these species in both niches, but also with regard to cell densities per bacterial species, confirming the possible role of the rectum as a reservoir for vaginal colonization. Using qPCR, we detected L. iners in high numbers both vaginally and rectally when compared to other studies of the rectal microflora (Antonio et al., 1999; Marrazzo et al., 2009). Several recent studies (Ferris et al., 2007; Jakobsson and Forsum, 2007) indicate that the role of this peculiar Lactobacillus species in the transition between balanced and imbalanced vaginal microflora warrants further elucidation.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.resmic.2011.04.004.

References


