ORIGINAL ARTICLE

A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm

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ABSTRACT

Objectives Bacterial vaginosis (BV) is characterised by a change in the microbial composition of the vagina. The BV-associated organisms outnumber the health-associated *Lactobacillus* species and form a polymicrobial biofilm on the vaginal epithelium, possibly explaining the difficulties with antibiotic treatment. A better understanding of vaginal biofilm with emphasis on *Atopobium vaginae* and *Gardnerella vaginalis* may contribute to a better diagnosis and treatment of BV.

Methods To this purpose, we evaluated the association between the presence of both bacteria by fluorescence in situ hybridisation (FISH) and BV by Nugent scoring in 463 vaginal slides of 120 participants participating in a clinical trial in Rwanda.

Results A bacterial biofilm was detected in half of the samples using a universal bacterial probe. The biofilm contained *A. vaginae* in 54.1% and *G. vaginalis* in 82.0% of the samples. *A. vaginae* was accompanied by *G. vaginalis* in 99.5% of samples. The odds of having a Nugent score above 4 were increased for samples with dispersed *G. vaginalis* and/or *A. vaginae* present (OR 4.5; CI 2 to 10.3). The probability of having a high Nugent score was even higher when a combination of adherent *G. vaginalis* and dispersed *A. vaginae* was visualised (OR 75.6; CI 13.3 to 429.5) and highest when both bacteria were part of the biofilm (OR 119; CI 39.9 to 360.8).

Conclusions Our study, although not comprehensive at studying the polymicrobial biofilm in BV, provided a strong indication towards the importance of *A. vaginae* and the symbiosis of *A. vaginae* and *G. vaginalis* in this biofilm.

Trial registration number NCT01796613.

INTRODUCTION

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in women of reproductive age. It increases the risk of acquisition and transmission of sexually transmitted infections, including HIV, and is associated with preterm birth in pregnant women.[1–3] The condition is characterised by a change in the microbial composition of the vagina: the *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by micro-aerophilic and anaerobic organisms, including *Gardnerella vaginalis*.[3–7] The mere presence of *G. vaginalis*, however, is not sufficient for the diagnosis of BV using traditional diagnostic algorithms (see below) because many women without BV also have *G. vaginalis* in their vaginal microbiome.[4] BV is, however, associated with high counts of *G. vaginalis* using molecular methods and/or the presence of a *G. vaginalis*-containing polymicrobial biofilm.[4–10] Due to its strong adherence to vaginal epithelial cells and biofilm-forming capacities, it has been suggested that *G. vaginalis* initiates the colonisation of the vaginal epithelium and serves as a scaffolding to which other species subsequently can attach.[10–12]

One of the species that might attach to the biofilm initiated by *G. vaginalis* could be *Atopobium vaginae*.[13–14] Several molecular studies have indicated a probable role for *A. vaginae* in BV,[14–16] and it has also been suggested that *A. vaginae* plays a major part in the establishment of a biofilm, together with *G. vaginalis*.[9 10] Considering it has been found in 80–90% of cases of relapse[17] and some strains have been shown in vitro to be metronidazole resistant,[18] it could be of importance in the recurrence of BV after standard treatment with metronidazole.

The current gold standard in BV research is the microscopic evaluation and scoring of vaginal slides according to Nugent.[19] The diagnosis of BV is based on the absence of lactobacilli and the presence of small Gram-negative to Gram-variable rods (*G. vaginalis* and *Bacteroides* spp. morphotypes) and curved Gram-negative rods (*Mobiluncus* spp. morphotypes). In fact, bacterial biofilm can also be seen with this method in the form of clue cells, which are vaginal epithelial cells covered by layers of adherent Gram-negative and/or Gram-variable cells, that is, biofilms.[20] Using Gram staining, it is impossible to distinguish between the different bacterial species in the biofilm. By labelling the cells with a fluorescent probe, using fluorescence in situ hybridisation (FISH), the structure and composition of the biofilm can be studied in more detail.

To study the potential role of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in the biofilm, we used our newly developed peptide nucleic acid (PNA) *A. vaginae* probe[11] together with an existing probe for *G. vaginalis*[21] and a universal bacterial probe[22] to investigate the composition of vaginal biofilm and its importance in BV.

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MATERIALS AND METHODS

Clinical samples

Vaginal sample collection and preparation

Vaginal samples were collected from 120 women participating in a clinical trial at Rinda Ubuzima in Kigali, Rwanda, studying the safety and acceptability of a contraceptive vaginal ring (Nuvaring), including the effect of the vaginal ring on the vaginal microbiome (the Ring Plus study—Clinicaltrials.gov NCT01796613).[23] Participants were between 18 and 35 years old and provided written informed consent for participation in the study. Depending on the group (continuous or intermittent ring use) to which the participant was randomised, a total of four or five samples from the same participant were taken over a period of four menstrual cycles. A total of 463 samples were analysed after Gram stain and after FISH using light microscopy and confocal laser scanning microscopy (CLSM), respectively.

Vaginal sampling was carried out by the study physician during a speculum examination in the Rinda Ubuzima research clinic. A cotton swab was brushed against the lateral walls of the vagina and was transported in its container to the Rinda Ubuzima laboratory within 20 min. Upon arrival in the laboratory, the swab was used to prepare a vaginal slide on a regular glass slide for Gram stain and a second vaginal slide on a Superfrost Plus slide (Menzel-Gläser, Braunschweig, Germany). All slides were air-dried, heat-fixed by passing through a flame twice and then stored in their appropriate boxes until Gram staining and/or shipment for FISH. The first slide was Gram stained and examined on-site in the Rinda Ubuzima laboratory in Kigali. The Superfrost Plus slides were stored and shipped at room temperature to the ITM where they were fixed for a minimum of 12 h in Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid).[11]

Microbiological analysis of the vaginal samples

Peptide nucleic acid fluorescence in situ hybridisation

PNA FISH was performed as described earlier[11] using species-specific probes for *A. vaginae* (AtoITM1) and *G. vaginalis* (Gard162) and the broad-range BacUni-1 probe. The hybridised samples were stored in the dark at room temperature for a maximum of 1 week before microscopic observation, using CLSM (LSM700, Zeiss, Oberkochen, Germany). Detection and identification of individual bacteria were done at 400× magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Separate scattered bacterial cells were defined as dispersed bacteria. Aggregates of bacterial cells, sticking to the vaginal epithelial cells, were defined as adherent bacteria forming a biofilm. The species-specific signal was considered positive only if it had a positive counterpart in the 4',6-diamidino-2-phenylindole (DAPI) stain and if it displayed a positive signal simultaneously with the universal probe. Semi-quantification was done for the dispersed and adherent bacteria in three categories (absent, present in low amount, present in high amount), but for the analysis only two categories (absent or present) have been used.

Nugent score

The status of the vaginal microbiome was assessed at the Rinda Ubuzima laboratory by Nugent scoring of a Gram stained vaginal slide.[19] A score of 0–3 was considered normal vaginal microbiome; a score of 4–6 intermediate microbiome and a score of 7–10 BV.

Statistical analysis

The clinical study sample size calculation was based on the primary objective to assess the pre–post changes in the vaginal microbiome and required 60 women in each group to require 95% power to detect clinically important changes in bacterial counts.[23] Data analysis was done using STATA10 (StataCorp LP, Texas, USA). While the samples were collected longitudinally, they were analysed cross-sectionally, with each sample as the unit of analysis. To study the association between the presence and absence of dispersed and/or adherent *A. vaginae* and adherent *G. vaginalis* in relation to BV status, we categorised the samples into five categories (table 1) based on combinations of the presence of both bacteria in dispersed and/or adherent form as visualised by FISH. To increase the statistical power, we made the vaginal microbiome status binary: Nugent score 0–3 (reference group) versus Nugent score 4–10 (table 2). A mixed-effects logistic regression model was fitted with BV as the binary outcome (ie, Nugent 0–3 vs Nugent 4–10) and biofilm characteristics as the main dependent variable. The model was adjusted for woman, randomisation group and study visit, because multiple samples per woman at multiple study visits were included in the analysis. ORs are reported with 95% CI and the p values are from χ^2 tests (table 2).

RESULTS

Characterisation of vaginal samples

In total, 463 of 527 samples from 120 women were available for FISH analysis, excluding 13 missing samples and 51 samples not readable due to the absence of epithelial cells on the slides. In all 463 samples, a positive signal was detected for the universal BacUni-1 probe. In 230 samples (49.7%), only dispersed bacteria were present, while the other 233 slides (50.3%) contained adherent bacteria as well (table 1). *A. vaginae* and *G. vaginalis* were part of this biofilm in 126 (54.1%) and 191 (82.0%) samples, respectively. Next, we visualised *A. vaginae* with FISH

Table 1 *Gardnerella vaginalis, Atopobium vaginae* and *G. vaginalis* with *A. vaginae* combinations for samples analysed with fluorescence in situ hybridisation (FISH) by absent, dispersed only and adherent ±dispersed category and stratified by Nugent scoring

	Total	Nugent 0–3	Nugent 4–6	Nugent 7–10
	N	N (%)	N (%)	N (%)
FISH all bacteria				
Absent	0	0 (0.0)	0 (0.0)	0 (0.0)
Dispersed only	230	197 (76.0)	19 (39.6)	14 (9.0)
Adherent ± dispersed	233	62 (24.0)	29 (60.4)	142 (91.0)
FISH *A. vaginae* (Av)				
Absent	268	201 (77.6)	24 (50.0)	43 (27.6)
Dispersed only	69	41 (15.8)	10 (20.8)	18 (11.5)
Adherent ± dispersed	126	17 (6.6)	14 (29.2)	95 (60.9)
FISH *G. vaginalis* (Gv)				
Absent	172	155 (59.8)	8 (16.7)	9 (5.8)
Dispersed only	100	71 (27.4)	15 (31.2)	14 (9.0)
Adherent ± dispersed	191	33 (12.8)	25 (52.1)	133 (85.2)
FISH Av and Gv combined				
Gv and Av absent	170	153 (59.1)	8 (16.7)	9 (5.7)
Gv or Av dispersed only	101	72 (27.8)	15 (31.2)	14 (9.0)
Gv adherent ± Gv dispersed and Av absent	51	14 (5.4)	8 (16.7)	29 (18.6)
Gv adherent ± Gv dispersed and Av dispersed	15	3 (1.1)	3 (6.2)	9 (5.8)
Gv and Av adherent ± Gv and Av dispersed	126	17 (6.6)	14 (29.2)	95 (60.9)

Table 2 Association between the bacterial presence of *Atopobium vaginae* and *Gardnerella vaginalis* by fluorescence in situ hybridisation (FISH) and the vaginal microbiome defined by Nugent scoring

G. vaginalis and *A. vaginae* combination	absent	*G. vaginalis* (Gv) or *A. vaginae* (Av) dispersed only	Gv adherent ± Gv dispersed and Av absent	Gv adherent ± Gv dispersed and Av dispersed only	Gv and Av adherent ± dispersed Gv and Av
Total=463	170	101	51	15	126
Nugent 0–3	153 (90)	72 (71.3)	14 (27.5)	3 (20)	17 (13.5)
Nugent 4–10	17 (10)	29 (28.7)	37 (72.5)	12 (80)	109 (86.5)
OR (CI)*	Reference	4.5 (2 to 10.3)	49.2 (15.9 to 151.8)	75.6 (13.3 to 429.5)	119 (39.9 to 360.8)
p Value χ^2 test*		0.001	<0.001	<0.001	<0.001

*The mixed-effects logistic regression model was adjusted for woman, randomisation group and visit.

in 195 (42.1%) samples; in 69 samples (14.9% of the total 463 samples) *A. vaginae* was present in a dispersed state, whereas in 126 samples (27.2%) the *A. vaginae* bacteria were seen adherent to epithelial cells (table 1). For 122 (97.0%) of the samples with adherent *A. vaginae*, concurrent dispersed *A. vaginae* bacteria were observed. *G. vaginalis* was detected by FISH in 291 (62.9%) samples; it was detected as dispersed-only *G. vaginalis* in 100 samples (21.6% of the total 463 samples) and for the remaining 191 samples (41.3%) *G. vaginalis* was adherent to the epithelial cells. Furthermore, when combining the results of both bacteria and considering only the 291 *G. vaginalis* FISH-positive samples, *A. vaginae* was absent in 98 of the slides (33.7%). On the contrary, only two (0.5%) of the 195 samples showing *A. vaginae* (dispersed and/or adherent) with FISH were negative for *G. vaginalis*; this included one sample with adherent *A. vaginae*.

One-third of the vaginal samples (n=156; 33.7%) was classified as Nugent score 7–10, 10% as Nugent score 4–6 (n=48; 10.4%) and the remaining 259 samples (55.9%) as Nugent 0–3. The majority of the samples without *A. vaginae* (n=201; 75.0%) and without *G. vaginalis* (n=155; 90.1%) were categorised as Nugent 0–3, thus indicating a healthy microbiome. A BV microbiome, defined by a Nugent 7–10 category, was present in 75.4% of samples with adherent *A. vaginae* (n=95) and in 69.6% of the slides with adherent *G. vaginalis* (n=133). In case of absent *G. vaginalis* and *A. vaginae* by FISH (n=170, 36.7%), a healthy microbiome (Nugent 0–3) was observed for 90.0% of the 170 samples (n=153). Furthermore, when considering *G. vaginalis* and *A. vaginae* adherent samples only (n=126), 75.4% of the samples were categorised as BV (Nugent 7–10) (FISH experiments in figure 1; table 1).

The presence of *A. vaginae*, *G. vaginalis* and combinations of both bacteria in dispersed and adherent forms in relation to BV status

The group of FISH samples without *A. vaginae* and *G. vaginalis* was used as the reference group (table 2). Compared with this reference group, the odds of having a Nugent score of 4–10 were increased when one or both bacteria were present in the dispersed state without adhering to the vaginal epithelium (OR 4.5 (CI 2 to 10.3)); it was increased further when *G. vaginalis* was part of an adherent biofilm on the epithelium (OR 49.2 (CI 15.9 to 151.8)) and even more when dispersed *A. vaginae* accompanied this *G. vaginalis* biofilm (OR 75.6 (CI 13.3 to 429.5)); ultimately the OR was highest when *A. vaginae* was part of the *G. vaginalis* biofilm as well (OR 119 (CI 39.9 to 360.8)).

DISCUSSION

We set out to study the potential role of *A. vaginae* in BV and the synergy between *A. vaginae* and *G. vaginalis* in the BV-associated biofilm.

Our study confirms that both *A. vaginae* and *G. vaginalis* are important constituents of the vaginal epithelial biofilm.[9 11] Adherent *A. vaginae* and *G. vaginalis* were visualised in, respectively, 54.1% and 82.0% of samples with bacterial biofilm (detected using the universal BacUni-1 probe), suggesting an important role for both bacteria in this polymicrobial biofilm. Using FISH, we only found two samples containing *A. vaginae* (dispersed in both, adherent in one) in the absence of *G. vaginalis*, while more than one-third of the *G. vaginalis*-positive samples was negative for *A. vaginae*. This is in accordance with prior reports on the association of *A. vaginae* with *G. vaginalis*.[9 11 15 16 24] We showed that the presence of both bacteria in the samples, regardless of their existence in a biofilm, was associated with an elevated or high Nugent score indicative for vaginal dysbiosis and BV. The highest probability of having a Nugent score higher than 3 was seen when both *A. vaginae* and *G. vaginalis* were part of a biofilm attaching to the vaginal epithelial cells.

The association of *G. vaginalis* with BV was originally described in 1954 by Gardner and Dukes.[25] The involvement of *A. vaginae* in BV, however, has only been established 10 years ago.[13–15] Swidsinski *et al*[9] found vaginal biopsies with vaginal biofilm to be positive for *G. vaginalis* and *A. vaginae* when using fluorescent probes, although in our hands this *A. vaginae* probe cross-reacted with other vaginal species as well.[11]

The presence of *A. vaginae* in the BV-associated biofilm could have a major impact on treatment. Susceptibility to metronidazole, the standard treatment for BV, varied significantly across various *A. vaginae* strains in vitro.[18] In vivo data are scarce, but Bradshaw *et al*[17] found that rates of recurrence of BV were higher when *A. vaginae* was present in the vaginal microbiome in addition to *G. vaginalis*. In another study with topical metronidazole gel by Ferris *et al*,[13] it was shown that a high concentration of *A. vaginae* before treatment was associated with complete or partial failure of treatment for BV. In the above studies, no distinction was made between dispersed and biofilm-associated bacteria. Nevertheless, as bacteria in a biofilm are less sensitive to antibiotic treatment[26] and considering the evidence from our study that the formation of a bacterial biofilm is more likely to occur when *A. vaginae* is present in the vaginal microbiome, future design of studies may want to take this distinction into account when treating BV.

Our study has shed new light on the significance of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in vaginal dysbiosis, using highly specific PNA probes for both bacteria. However, a limitation was that we used multiple samples from the 120 women of the Ring Plus study. Ideally, we should repeat the study in a larger group of women. Furthermore, although we assessed the association between bacterial biofilm and vaginal dysbiosis, more research is needed to

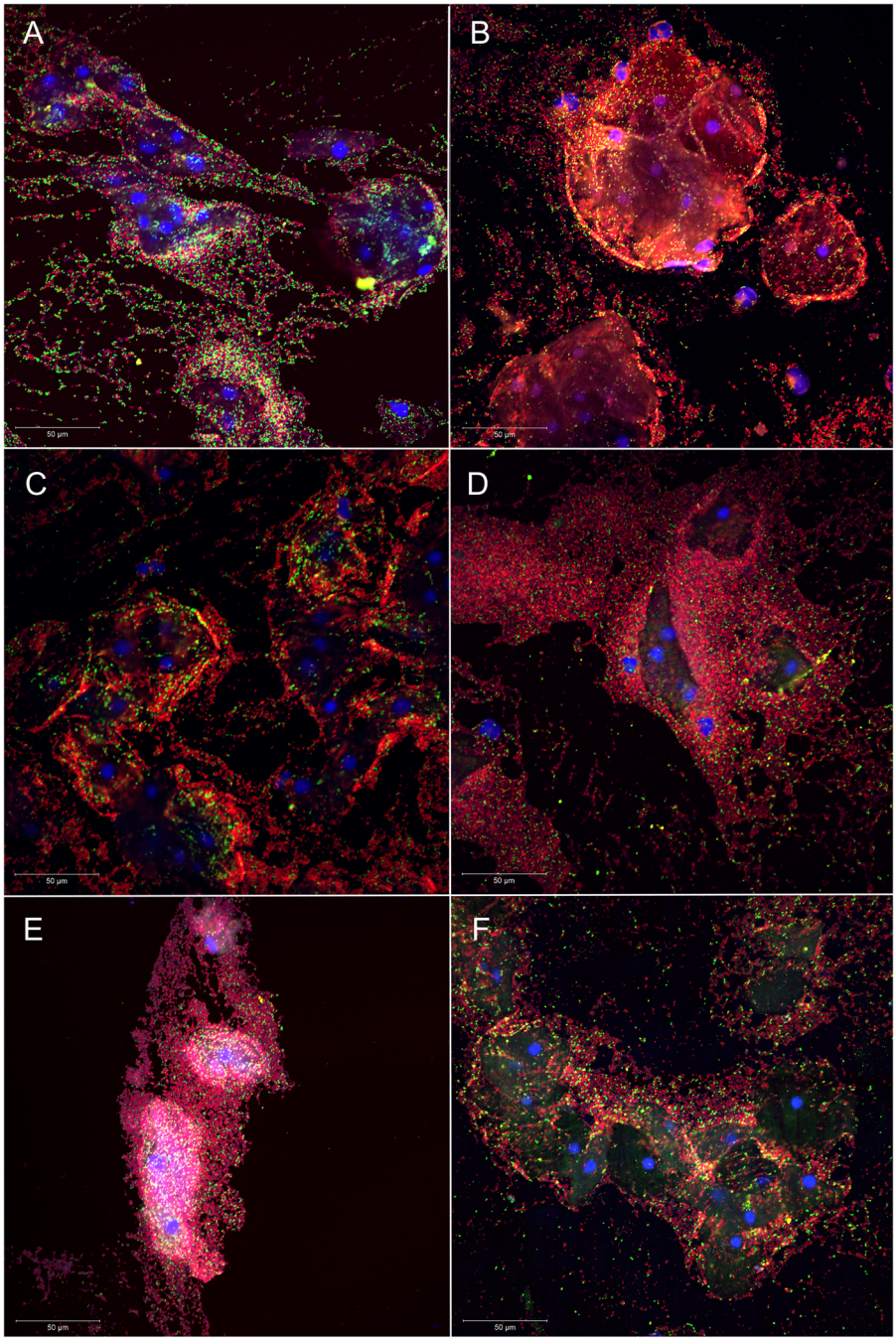

Figure 1 Superimposed confocal laser scanning images with 400× magnification of *Atopobium vaginae* +*Gardnerella vaginalis* biofilm in six vaginal samples (A–F): vaginal epithelial cells DAPI in blue, *A. vaginae*-specific peptide nucleic acid (PNA)-probe AtoITM1 with Alexa Fluor 488 in green and *G. vaginalis*-specific PNA-probe Gard162 with Alexa Fluor 647 in red. For clarity, we omitted the BacUni-1 plane, such that the bacteria that did not hybridise with Gard162 and AtoITM1 are visible in DAPI blue only.

unravel the exact mechanisms of biofilm formation in BV, including the role and the importance of both bacteria studied, to finally define improved regimens for treatment of BV. Moreover, since BV is a polymicrobial condition, new research should study the involvement of other bacteria related to BV.

In conclusion, the presented study uncovered a key piece of the BV puzzle confirming first, the importance of *A. vaginae* in BV-associated biofilm and second, showing the joint presence of *A. vaginae* and *G. vaginalis* in a biofilm. Future studies covering a wide array of BV-associated bacteria may help to further delineate biofilm mechanisms in BV.

Key messages

- This study shows that *Atopobium vaginae* is an important constituent of the vaginal biofilm, and is of relevance in the context of bacterial vaginosis (BV).
- We show that *A. vaginae* is almost always accompanied by *Gardnerella vaginalis* in BV, but that *G. vaginalis* can be found without *A. vaginae* in the vaginal microbiome.
- By tackling constituents of the biofilm, the above knowledge can contribute to a more effective and goal-oriented treatment and improve women's reproductive health.

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Contributors All authors were involved in the main study that generated the data. For the present study, LH wrote the first draft of the manuscript. VJ, TC, MV and JvdW revised and edited the text. IDB, TC, LH, VJ and JvdW created the experimental design. SA, LM, VM and LH performed the testing and VJ and LH performed the data analysis. All authors revised and approved the present version of the manuscript.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The Ring Plus study was approved by the Rwanda National Ethics Committee, Rwanda (Approval number 481/RNEC/2013); the ethics committees of the Institute of Tropical Medicine (ITM), Belgium (Approval number 864/13); the Antwerp University Hospital, Belgium (Approval number 13/7/85) and the University of Liverpool, UK (Approval number RETG000639IREC).

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