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Research article

Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis

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Abstract

Background: The pathogenesis of bacterial vaginosis remains largely elusive, although some microorganisms, including *Gardnerella vaginalis*, are suspected of playing a role in the etiology of this disorder. Recently culture-independent analysis of microbial ecosystems has proven its efficacy in characterizing the diversity of bacterial populations. Here, we report on the results obtained by combining culture and PCR-based methods to characterize the normal and disturbed vaginal microflora.

Results: A total of 150 vaginal swab samples from healthy women (115 pregnant and 35 non-pregnant) were categorized on the basis of Gram stain of direct smear as grade I (n = 112), grade II (n = 26), grade III (n = 9) or grade IV (n = 3). The composition of the vaginal microbial community of eight of these vaginal swabs (three grade I, two grade II and three grade III), all from non-pregnant women, were studied by culture and by cloning of the 16S rRNA genes obtained after direct amplification. Forty-six cultured isolates were identified by tDNA-PCR, 854 cloned 16S rRNA gene fragments were analysed of which 156 by sequencing, yielding a total of 38 species, including 9 presumptively novel species with at least five species that have not been isolated previously from vaginal samples. Interestingly, cloning revealed that *Atopobium vaginae* was abundant in four out of the five non-grade I specimens. Finally, species specific PCR for *A. vaginae* and *Gardnerella vaginalis* pointed to a statistically significant co-occurrence of both species in the bacterial vaginosis samples.

Conclusions: Although historically the literature regarding bacterial vaginosis has largely focused on *G. vaginalis* in particular, several findings of this study – like the abundance of *A. vaginae* in disturbed vaginal microflora and the presence of several novel species – indicate that much is to be learned about the composition of the vaginal microflora and its relation to the etiology of BV.

Background

Bacterial vaginosis (BV) is considered to be the most common cause of vaginal inflammation among both pregnant and non-pregnant women and prevalences between 4.9% and 36.0% have been reported from European and American studies [1]. The etiology of this condition remains largely unknown.

Nonetheless, the abundant literature, addressing bacterial vaginosis and ascending genital tract infection, has largely focused on the bacterial component and, in particular, on a few microorganisms, which are thought to play a pivotal role in the pathology of bacterial vaginosis. Although probably rather complex microbial community dynamics are involved, culture-dependent studies indicate that a limited number of bacterial species, including *Mobiluncus* spp., *Gardnerella vaginalis*, *Bacteroides* spp., *Prevotella* spp. and *Mycoplasma hominis*, along with the relative absence of *Lactobacillus* spp., can be used as a sensitive index for bacterial vaginosis. Consequently, according to Nugent *et al*. [2], the standard diagnosis of BV relies on the quantification of only three cellular types (*Lactobacillus*, *Gardnerella*, and *Mobiluncus*) on a Gram stained vaginal smear.

While conventional microbiological techniques are useful as screening tools to identify women with BV, they do not enable prediction of the clinical burden associated with bacterial vaginosis. It has therefore been stated that better understanding of the composition and dynamics of the vaginal microflora along with the factors associated with the pathology of bacterial vaginosis is essential to improve our predictive abilities [1].

The analysis of complex bacterial communities has been hampered by conventional culture-dependent methods and by biochemical identification methods, since it leaves many bacteria uncultured and unidentified. This may prove especially true for vaginal microflora, both under normal circumstances as well as in the setting of bacterial vaginosis, a condition primarily characterized by overgrowth of anaerobic and fastidious microorganisms. For example, until recently, several important species, like *Lactobacillus crispatus*, *L. gasseri* and *L. iners*, were all lumped together into the *L. acidophilus* complex, while present molecular techniques make it possible to differentiate between these closely related species [3-9]. Here we combined culture with tDNA-PCR [4,10,11] and sequencing of the 16S rRNA-gene for the identification of cultured organisms.

Detailed information of complex microbial communities can also be acquired from the phylogenetic analysis of 16S rDNA sequences obtained directly from samples by PCR amplification, cloning, and sequencing, so that uncultivable species are also included [12,13], although this approach may be biased as well [14,15], as becomes also apparent from this study.

Here we report on the cloning and sequencing of 16S rRNA gene fragments, amplified directly from vaginal swabs, to examine the microbial diversity in the vaginal fluid of healthy women with different grades of vaginal microflora patterns as defined previously [16,17]. Finally, the data obtained by anaerobic culture and with culture independent techniques, lead us to carry out species specific PCR for *A. vaginae* and *G. vaginalis*, such that the presence of both species in differently graded samples could be established.

Results

Grading of the Gram stained smears of the vaginal samples for which cloning was carried out, according to the criteria of Ison and Hay [17] assigned three specimens (W1-W3) to grade I, two (W4-5) to grade II and three (W6-8) to grade III. Table 1 lists the species identified by cloning and sequencing of cloned bacterial 16S rDNA-fragments, as well as by bacterial culture and tDNA-PCR from the vaginal microflora of these 8 healthy women.

Identification of cultured isolates from 8 healthy women by tDNA-PCR and 16S rRNA gene sequencing

Forty six isolates were obtained after anaerobic culture of the vaginal fluid from the 8 women in the cloning part of the study. Twenty nine isolates, belonging to the species *Bacteroides ureolyticus*, *Enterococcus faecalis*, *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri*, *L. vaginalis*, *Peptoniphilus* sp., *Prevotella bivia*, *Streptococcus anginosus* group and *S. mitis* group were identified by tDNA-PCR using a library containing tDNA-PCR fingerprints of well-identified strains. Six isolates belonging to the species *Aerococcus christensenii*, *Anaerococcus tetradius*, *Anaerococcus vaginalis*, *Lactobacillus iners*, *Mobiluncus mulieris* and *Peptostreptococcus anaerobius* were identified by sequencing because they produced a tDNA pattern that initially was not present in the database. These species were characterized by a specific tDNA-PCR pattern that allowed unambiguous identification after the new patterns were added to the database. In summary, 40 of 46 isolates (87.0%) were correctly identified by tDNA-PCR to the species level, including the species within the *L. acidophilus* complex. Of the six isolates that could not be identified, one isolate, with a sequence that was 99% identical to that of a *Peptostreptococcus* sp. strain (CCUG 42997, AJ277208), could not be identified unambiguously by tDNA-PCR because its pattern was identical to that of *P. micros*, one isolate – that was identified by sequencing as *Atopobium vaginae* – did not yield a tDNA-pattern and another four isolates (8.7%) were tDNA-PCR negative and also 16S PCR negative.

Table 1: Cloning and culture[a] results for 8 healthy females with different grades of vaginal microflora.

Grade	**I**	**I**	**I**	**II**	**II**	**III**	**III**	**III**
Sample designation	**BVS30**	**BVS62**	**BVS63**	**BVS36**	**BVS34**	**BVS59**	**BVS61**	**BVS44**
Subject code	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**	**W7**	**W8**
Age	51	34	38	49	41	46	28	44
Number of clones	124	118	107	69	72	125	169	70
Species								
Lactobacillus crispatus [AF257097][b]	66.1[c]		99.1					
Lactobacillus gasseri [AF243144]	18.5	99.2						
Lactobacillus jensenii [AF243159]			0.9					
Lactobacillus vaginalis [AF243177]		0.8						
Atopobium vaginae [AF325325]				1.4	41.7	36.0	80.5	
Atopobium vaginae 97.6%[d] [AF325325]						0.0		
Gardnerella vaginalis[e] [M58744]				0.0		0.0	4.1	
Lactobacillus iners [Y16329]				84.1	1.4		12.4	
Mobiluncus mulieris [AJ427625]						5.6	3.0	
Peptostreptococcus anaerobius [L04168]					0.0	1.6		68.6
Peptoniphilus sp. [D14147]						0.0		14.3
Prevotella bivia 91% [L16475]				7.2	22.2			
Prevotella buccalis 96.6% [L16476]	1.6				22.2	31.2		
Sneathia (Leptotrichia) sanguinegens [L37789]				1.4	1.4	6.4		
Uncultured *Megasphaera* sp. clone [AY271937]				1.4		4.8		
Uncultured *Actinobacteridae* clone 86% [AB089070]				2.9		0.8		
Unidentified clone 1[f] [AY207059]					6.9	4.0		
Atopobium rimae [AF292371]	0.8							
Fusobacterium nucleatum [AJ006964]	6.5							
Peptostreptococcus sp. [AJ277208]	0.8							
Pseudoramibacter alactolyticus [AB036761]	0.8							
Streptococcus anginosus group [AF104676]	0.0							
Treponema sp. clone [AF023055]	0.8							
Porphyromonas levi clone 94% [L16493]	1.6							
Unidentified clone 2 [AF371910]	2.4							
Aerococcus christensenii [Y17318]						3.2	0.0	
Anaerococcus tetradius [AF542234]							0.0	
Anaerococcus vaginalis [AF542229]					1.4			
Bacteroides ureolyticus [L04321]							0.0	
Bifidobacterium biavatii (urinalis) [AJ278695]								1.4
Dialister sp. [AF473837]								2.9
Enterococcus faecalis [AJ420803]					1.4			
[Leptotrichia amnionii] [AY078425]					1.4			
Prevotella bivia [L16475]				0.0	0.0			
Prevotella ruminicola 87% [L16476]								4.3
Streptococcus sp. oral strain [AY005041]								8.6
Unidentified clone 3 [AF371693]						6.4		
Ureaplasma urealyticum [AF073455]				1.4				

[a] Four isolates were cultured which were negative for amplification of the 16S rRNA gene and for tDNA-PCR. [b] The accession number of the Genbank sequence with the highest similarity is indicated within brackets. [c] Underlined figures indicate that this species was also cultured. [d] Percentage of 16S rRNA gene sequence similarity, given only for values below 98%. [e] Low clone numbers of *G. vaginalis* are caused by the usage of one primer with 3 mismatches for this species (see Methods). [f] The 16S rRNA gene sequences of these clones showed highest similarity with entries in the Genbank listed as being obtained from uncultured bacteria.

Identification of 16S rRNA gene clones from 8 healthy women by sequencing and culture results

The ARDRA pattern of 854 clones was analysed and enabled to establish the relative frequency of the different species present (Table 1). For 130 clones, belonging to 33 ARDRA types, on average 447 bp of the 5' end of the 16S rRNA gene was sequenced. In case of grade I vaginal smears, the most predominant species were *L. crispatus*

and *L. gasseri*. Smaller numbers of *L. jensenii* and *L. vaginalis* were also present. All females with grade I smears (W1-W3) were colonized by two *Lactobacillus* species. W2 (age 34) and W3 (age 38) were colonized by *Lactobacillus* species only, with one species – respectively *L. gasseri* and *L. crispatus* – represented by more than 99% of the clones. In the vaginal fluid of W1 (age 51) the most abundant species were *L. crispatus* (66.1%), *L. gasseri* (18.6%) and *Fusobacterium nucleatum* (6.5%). An additional 8 species were found in low numbers, six only by cloning and one only by culture. Three of these species, representing 5.6% of the clones, showed less than 98% similarity with previously published sequences. Culture results coincided largely with cloning for the grade I samples, except for the non *Lactobacillus* species of W1 of which only two were cultured.

For the two grade II smears (W4 and W5) cloning revealed four species, *A. vaginae* (resp. 1.5% and 41.6%), *L. iners* (resp. 84.1% and 1.4%), a *P. bivia*-like species (resp. 7.3% and 22.2%) and *Sneathia sanguinegens* (both 1.4%), that were present in both samples. *P. bivia* was also present in both grade II microflora but it was found by culture only. Several other species were found by cloning only in either W4 or W5. For example, *[Leptotrichia amnionii]* was found only in W5. *G. vaginalis* and *Peptostreptococcus anaerobius* were found respectively in W4 and W5 by culture only and two species, *Anaerococcus vaginalis* and *Enterococcus faecalis*, were found by both cloning and culture in the microflora of W5.

Two women (W6 and W7) with a grade III vaginal smear were colonized predominantly by *A. vaginae* (resp. 36.0 and 80.5%), *L. iners* (absent and 12.4%), *G. vaginalis* (absent and 4.1%), *M. mulieris* (5.6 and 3.0%) and a *Prevotella buccalis*-like species (31.2% and absent), according to cloning. As for grade II samples, there was limited correspondence with culture. For W6, an *A. vaginae*-like organism was cultured, but the sequence of this isolate showed less than 98% similarity to the sequence of the clones. Furthermore, a large number of colonies of *G. vaginalis* and *Peptoniphilus* sp. were present after anaerobic culture but no clones were obtained.

The most abundant species obtained by cloning the third grade III vaginal sample (W8) were *Peptostreptococcus anaerobius* (68.6%)(also cultured), *Peptoniphilus* sp. (14.3%) and an unidentified *Streptococcus* sp. (8.6%)(also cultured).

Total number of species identified and comparison between cloning and culture

Of the 38 species, 18 were discovered by cloning only, 5 by culture only, and 8 by both cloning and culture. Three species were shown by both culture and cloning in some samples, but only by culture in the remaining samples, another three species were shown by both cloning and culture in some samples, but only by cloning in the remaining samples and one species was found once by culture only and once by cloning only. The presence of all four grade I *Lactobacillus* sp. was shown in grade I samples by both cloning and culture, whereas *L. iners* was found only in three non-grade I samples, in all three by cloning – even abundantly in W4, but only in W7 by culture. *G. vaginalis* was cultured three times, but was found by cloning only once. *A. vaginae* was shown in four samples by cloning, but cultured only once. The *P. bivia*-like species and *P. buccalis*-like species were found in respectively two and three samples by cloning but not by culture.

Possible novel species and genera

Of the 38 species that were distinguished by culture and cloning in this study, only 76.3% demonstrated more than 98% identity with previously known bacterial species (Table 1). Five of the eight vaginal samples contained previously unidentified species.

Anaerobic culture of the microflora of W6 revealed an *Atopobium* species that showed only 97.6% similarity with previously reported *A. vaginae* sequences (see below).

Two presumptively novel species within the genus *Prevotella* were found. For W4 and W5 (grade II), respectively 7.3% and 22.2% of the clones had 91% similarity with a *P. bivia* sequence. For W1 (grade I), W5 and W6 (grade III), respectively 1.6%, 22.2% and 31.2% of the clones showed 96.6% similarity with a *P. buccalis* sequence. Respectively 2.9% and 0.8% of the clones of W4 (grade II) and W6 (grade III) were identical and had only 86% similarity with an uncultured termite Actinobacteridae bacterium (accession number AB089070).

Variation in Atopobium *sequences*

Sixteen *A. vaginae* sequences are present in GenBank, one from a clinical isolate [18], one from the type strain [19] and 14 from cloned 16S rDNA fragments (Zhou, unpublished) (Figure 1). Another 17 sequences were obtained in this study, one from an isolate from patient W6, two from isolates from other patients (BVS38 and PB9) and 14 from clones from four different patients, i.e. W4 (grade II), W5 (II), W6 (III) and W7 (III) (Figure 1).

The sequence of all 7 clones from W5 was different from that of all other isolates and from the clones and the Genbank sequences, except from one Genbank clone sequence (AY269023). The sequence of all clones from the three other patients and from the two isolates from samples BVS38 and PB9 were identical to the previously published clinical isolate [18] and the type strain [19]. The sequence of the cultured isolate from patient W6 was

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Genbank  Study                          Sequence
AF325325 [15]                          ATACTCCATA TTTGTCGCAT GGCGAATATG GGAAAGCTCC GGCGGCAAAG
Y17195 (Type strain) [16]              .......... ...N...... .......... .......... ..........
ato167f*                                                    ......... .......... .
ato154f*                                      ... .......... .......
AJ585206 this study, W6 isolate 5      .......... .A.A...... .AT.T..... .......... .A........
AJ585208 this study, W6 clone 1        .......... .......... .......... .......... ..........
AJ585209 this study, W7 clone 4        .......... .......... .......... .......... ..........
AJ585212 this study, W7 clone1         .......... .......... .......... .......... ..........
AJ585213 this study, W7 clone9         .......... .......... .......... .......... ..........
AJ585210 this study, W5 clone 41       .......... .CCAC..... .ATAG..... .......... ..........
AJ585211 this study, W5 clone 47       .......... .CCA...... .ATAG..... .......... ..........
AY269023 (Zhou, unpublished)           .......... .CCA...... .ATAG..... .......... ........-.
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Figure 1
***Atopobium vaginae* 16S rDNA sequences** * Designations of the forward primers specific for *A. vaginae* and their position in the 16S rRNA gene.

Table 2: Amplification results with *A. vaginae* and *G. vaginalis* specific primers obtained for 150 vaginal samples of different grades

Grade	n	A+[a] G_{OY}+/G_Z+	A+ G_{OY}-/G_Z-	A- G_{OY}+/G_Z+	A- G_{OY}-/G_Z-
I	112	2/11	20/11	3/21	87/69
II	26	3/4	6/5	5/9	12/8
III	9	7/7	0/0	0/1	2/1
IV	3	0/0	0/0	0/1	3/2
total	150	12/22	26/16	8/32	104/80

[a] A: *A. vaginae* amplified with ato167f, G_{OY}: *G. vaginalis* amplified with Obata-Yasuoka primer set [25], G_Z: *G. vaginalis* amplified with Zariffard primer set [26].

somewhat intermediate between both above sequences, although the sequence of the clones of the same patient were identical to that of the type strain. Specific amplification with both the ato167f primer and the ato154f primer gave positive signals for the vaginal sample of W6, indicating that indeed both types of sequence were present.

Species-specific PCR for A. vaginae *and* G. vaginalis

To substantiate the results obtained by cloning, an additional series of 142 vaginal samples obtained by swab from healthy pregnant (n = 115) and non-pregnant (n = 27) women, attending our out-patient clinic, were selected for culture and for PCR with 16S rRNA gene based primers specific for *A. vaginae* and *G. vaginalis* (Table 2). Of the 150 subjects in total, 38 (of which 5 non-pregnant) presented with non-grade I microflora, of which 26 were assigned grade II, 9 grade III and 3 grade IV.

After amplification with the ato167f *A. vaginae* primer set, respectively 19.6% of grade I, 34.6% of grade II, 77.8% of grade III and 0.0% of grade IV samples showed an amplicon (Table 2). Of the 37 samples that showed an amplicon after amplification with the ato167f primer only 23 were positive after amplification with ato154f. *A. vaginae* was cultured only three times from these 150 samples.

The number of positive samples for *G. vaginalis* specific PCR varied depending on the primer set that was used. The difference was greatest for grade I samples, with respectively 4.5% of grade I samples that were positive after amplification with the G_{OY} primer set and 28.6% with the G_Z primer set (Table 2).

Comparative ROC analysis of the four indicators (ato154f, ato167f, G_Z, and G_{OY} respectively) in discriminating normal and disturbed vaginal microflora showed that the overall discriminative value of qualitative PCR-based detection of *A. vaginae* or *G. vaginalis* as such is actually rather low with AUCs of 0.627 (95%CI: 0.544–0.704), 0.625 (95%CI: 0.543–0.703), 0.633 (95%CI: 0.551–0.711), and 0.675 (95%CI: 0.594–0.749).

Detection of the simultaneous presence of *A. vaginae* (ato167f) and *G. vaginalis* (G_{OY}) in a vaginal swab specimen with the purpose of assessing true bacterial vaginosis (grade III) had an accuracy of 87.8% (AUC = 0.878, 95%CI = 0.714, 1.041, $p < 0.001$), a sensitivity of 0.78 (95%CI = 0.40, 0.96), a specificity of 0.98 (95%CI = 0.91, 1.00), a positive predictive value of 0.78 (95%CI = 0.40, 0.96), and a negative predictive value of 0.98 (95%CI = 0.91, 1.00). When the co-existence of *A. vaginae* (ato167f)

and *G. vaginalis* was assessed using the G_Z-primer set, the overall performance of the assay was significantly lower.

Discussion

To our knowledge, no cloning study addressing the composition of the microflora of vaginal samples and bacterial vaginosis samples has been published thus far. By means of culture and by cloning and sequencing of 16S rRNA genes, amplified directly from the vaginal samples, we studied the vaginal microflora of 8 healthy women with different grades of bacterial vaginosis according to Nugent [2], as modified by Ison and Hay [16,17].

The frequency of the different clones could be assessed by performing ARDRA of the cloned 16S rRNA genes and counting the number of clones belonging to each ARDRA type. Representative clones for each ARDRA type were subsequently sequenced to determine the species identity. Five bacterial species, i.e. *Atopobium rimae*, *Bifidobacterium biavatii (urinalis)*, *Dialister* sp., *[Leptotrichia amnionii]* and *Sneathia sanguinegens*, had never been recovered from the vagina. Twenty two percent of the clones, belonging to 9 putative species, showed less than 98% homology to any of the 16S rRNA gene sequences present in GenBank, indicating that several bacterial species, indigeneous to the female genital tract, remain to be characterized.

In this study, cloning confirmed that the vaginal microflora of healthy women is dominated by a limited number of *Lactobacillus* species. For the women with grade I microflora, the two species *L. crispatus* and *L. gasseri*, alone or in combination, accounted for 85–99% of the clones. The two younger women, 34 and 38 years, had almost pure cultures of *L. crispatus* resp. *L. gasseri*, in association with low numbers of resp. *L. jensenii* (< 1%) and *L. vaginalis* (< 1%) and without any other bacteria. In our ongoing studies we find this to be the case for 61.5% of the grade I samples (unpublished data). The finding of monocultures of *L. crispatus* and *L. gasseri* is also in correspondence with recent reports, which point to the predominance of only three to four *Lactobacillus* species in normal vaginal microflora [7,20]. Antonio *et al*. [3] considered *L. crispatus*, *L. jensenii* and *L. gasseri* as the indicator species for normal vaginal microflora, also because most strains of these species are hydroxyperoxide producers in opposition to other *Lactobacillus* spp. It should be noted in this context that another group could not substantiate any protective effect of hydroxyperoxide producing lactobacilli [21].

In the 51-year-old woman (W1), with a combination of both *L. crispatus* and *L. gasseri*, some additional non *Lactobacillus* species were present in low numbers, with *Fusobacterium nucleatum* as the most predominant (i.e. 6.5%). This may be in accordance with another observation, namely that postmenopausal healthy women frequently present with BV-like microscopy [5,22].

Of the five women with non-grade I microflora (two with grade II and three with grade III), one presented with predominant *Peptostreptococcus* clones (68%), a picture that was very different from that observed in the other women. However, most striking was the observation that for the other four patients between 1.5% and 80.0% of the clones were identified as *Atopobium vaginae*, a species previously known only from a single isolate from the vagina of a healthy woman [19] and from a clinically important isolate, described as the causative agent in a case of a pelvic inflammatory disease (PID) following transvaginal oocyte recruitment [18]. Interestingly, Zhou *et al*. [20] reported dominating *Atopobium* sp. in a woman, judged to carry a normal vaginal microflora. The authors concluded that *Atopobium* may be present as part of the normal microflora of the vagina [23]. This warrants a more detailed discussion of this recently described species.

The genus *Atopobium* was introduced to accommodate the species *Lactobacillus minutus*, *Lactobacillus rimae* and *Streptococcus parvulus* [24]. *Atopobium* species have been described to produce major amounts of lactic acid [19], a characteristic reminiscent of lactobacilli, although no special reference to this property was made in the description of *A. vaginae* [19]. *Atopobium* species are anaerobic, Gram-positive elliptical cocci or rod-shaped organisms occurring singly, in pairs or as short chains. The variable cell morphology of *A. vaginae* makes that this species may reside perfectly camouflaged and as a consequence undetectable among the mixture of other species present in grade II and III bacterial communities. Also the fact that *A. vaginae* is fastidious and forms small pinhead colonies can explain why this species was not yet established as part of the vaginal microflora, when using classical microbiology. In this study, we could culture the species from only three out of 150 vaginal specimens.

Based on our findings with *A. vaginae* specific PCR, which recovered this species in 19.6% of the 112 grade I specimens, it appears as if *A. vaginae* may be a constituent – presumably in low numbers – of the human vagina, possibly attaining replicative dominance in association with decreasing lactobacillary grading. This hypothesis could be substantiated with quantitative PCR and the development of selective culture media.

The striking fact that *Gardnerella vaginalis*, predominantly present in bacterial vaginosis samples [e.g. [25,26]], was not found by cloning as carried out in this study, can be explained as the consequence of a methodological bias, because the forward primer used for cloning (10f) contained 3 mismatches for *G. vaginalis*. On the other hand,

the use of these non *G. vaginalis* 'universal' primers may have facilitated to establish the high prevalence of *A. vaginae*. To assess the relative importance of both species, cloning should be carried out with different sets of primers. Amplification of grade I, grade II and grade III samples with species specific primers indicated that *G. vaginalis* was present in respectively 28.6%, 50.0 % and 88.9% of the samples, while *Atopobium* was present in respectively 19.6%, 34.6% and 77.8% of the vaginal microflora.

Since any pairwise comparison of the four ROC plots did not show any significant differences between the AUCs, it is also apparent that each of the four indicators under study (ato154f, ato167f, G_Z, and G_{OY} respectively) presents with a comparably limited accuracy in discriminating normal and disturbed vaginal microflora. This may be explained by the fact that, although both species are present in about 80% of the true bacterial vaginosis samples, their presence in grade I samples is not uncommon. This lack of accuracy for *G. vaginalis* was also previously assessed in culture-dependent studies [27,28].

The co-existence of *A. vaginae* and *G. vaginalis* in women with grade III microflora together with their simultaneous absence in grade I samples (Table 2) is striking. The simultaneous presence of both species therefore is highly predictive for BV.

Lactobacillus iners, was present among the clones in three of the five non-grade I samples, even in large numbers, and absent from the three grade I samples. This species was only recently recognized as a separate *Lactobacillus* species [29]. In the original description of this relatively asaccharolytic *Lactobacillus* species, the only vaginal isolate among the 9 strains from females came from vaginal discharge [29]. This species may have been largely overlooked in culture based studies [30-32] since it does not grow on *Lactobacillus* selective media, including MRS and Rogosa-Sharp medium [29]. Using molecular techniques *L. iners* was reported as one of the most frequently encountered vaginal *Lactobacillus* species [8,9] present in normal and BV microflora [5].

Leptotrichia sp. are slow-growing, gram-negative anaerobic organisms of the oral cavity and genital tract. [*L. amnionii*] is an extremely fastidious organism, which most likely explains why this organism was hitherto virtually undetectable by conventional culture-based microbiological techniques. Of interest is that this particular *Leptotrichia* species was isolated by Shukla *et al*. [33] from the amniotic fluid of a woman with second trimester fetal loss, which led the authors to conclude that *[L. amnionii]* is presumably indigenous to the genital tract and acts as an opportunist under particular clinical conditions, including pregnancy. This study demonstrates for the first time the presence of *[L. amnionii]* in the vagina.

Sneathia sanguinegens (formerly *Leptotrichia sanguinegens*) was found in this study to be present in three patients with non-grade I vaginal microflora, in moderate numbers. *S. sanguinegens* has been isolated from human blood and amniotic fluid [34] and has been associated with several cases of pregnancy-associated bacteraemia, i.e. postpartum fever in four patients and neonatal sepsis in two patients [35]. This study demonstrates the presence of *S. sanguinegens* in the vagina for the first time.

One strain from W6 and 14.3% of the clones from W8 were identified as *Peptoniphilus indolicus* based on more than 98% similarity with a Genbank sequence with accession number D14147 corresponding with the type strain CCUG 17639 of this species. This type strain however has two Genbank entries (also AY153430) with low similarity [36]. Unfortunately our isolate was not stored so further phenotypic identification was not possible. Because of the ambiguous Genbank sequences, we chose to designate our isolate and clones as *Peptoniphilus* sp.

Thus far, identification of organisms cultured from complex microbial biota like the vagina, especially under the condition of vaginosis, has also been hampered by the limitations of conventional biochemical and phenotypic identification methods. However, using a DNA-based method, namely tDNA-PCR [11], it is convenient to appropriately identify most of these organisms, once a library, based on tDNA-PCR of well characterized organisms, has been constructed. Also, newly unknowns can be first identified by 16S rRNA gene sequence determination, whereafter it is possible to use the corresponding tDNA fingerprint for future identification of the organisms of the same species.

tDNA-PCR, which consists of the amplification of the spacer regions between tRNA genes, has been shown to yield mainly species-specific DNA fingerprints [10]. Combined with capillary electrophoresis, tDNA-PCR has been shown to be a semi-automated, digital DNA-fingerprinting method that enables rapid and discriminatory identification of species from very diverse phylogenetic groups, including *Lactobacillus* [4,11].

Conclusions

In summary, the use of tDNA-PCR based identification of cultured organisms in combination with cloning of 16S rRNA genes, amplified directly from vaginal swabs, enabled us to characterize the vaginal microflora in a detailed manner, and to compare the composition of the normal vaginal microflora with the bacterial vaginosis microflora. The presence of *A. vaginae* in 4 of the 5 women with bac-

terial vaginosis grade II-III microflora is an unexpected and previously not reported finding, which may shed new light on the etiology of this condition. There is also the ambiguous position of *L. iners*, which apparently is not indicative of a normal microflora, but may point to some intermediate condition.

Furthermore, our findings warrant more detailed studies of the ability of species like *L. iners* and *A. vaginae* to produce lactic acid, hydrogen peroxide and of their cellular and colonial morphology and biochemical characteristics, as well as their interaction with lactobacilli and *G. vaginalis*. A selective medium for these organisms might be a welcome tool for further studies, given the fact that until now they have been largely overlooked by culture methods.

Finally, the discrepancies between culture, cloning and specific PCR, together with the diversity of the recovered species, their unequal distribution over different vaginal samples, the elucidation of several species – including presumptively new species – previously not associated with the vagina, indicate that much is to be learned about the composition of the vaginal microflora and its relation to the etiology of BV.

Methods

Study population, sample collection and grading

For a total of 150 healthy women of reproductive age, attending our out-patient clinic, of which 115 were pregnant, the health condition with regard to the composition of the vaginal bacterial community was assessed microscopically after Gram stain, according to the criteria of Hay and Ison [17] and vaginal samples were taken. Briefly, specimens were considered grade I (normal) when only *Lactobacillus* morphotypes were present, grade II (intermediate) when both *Lactobacillus* and other morphotypes were present, grade III (BV) when only non *Lactobacillus* morphotypes were seen and grade IV when only Gram positive cocci were seen.

Sampling was carried out as follows. After placement of a non-lubrificated speculum two sterile cotton swabs were inserted into the vaginal vault. The swabs were rotated against the vaginal wall at the midportion of the vault and were carefully removed to prevent contamination with the vulva and introitus microflora. One swab was returned to a sterile tube (dry swab) (Copan, Brescia, Italy), for the purpose of DNA-extraction. The other swab was placed into Amies transport medium (Nuova Aptaca, Canelli, Italy) and was used for making a smear for the purpose of grading according to the Hay and Ison criteria [17] and for anaerobic culture. Both swabs were processed in the microbiology laboratory within 4 h.

Based on smear results, eight non-pregnant women (mean age 41.4 years, range 28–51 years) from the studied population, attending our out-patient clinic for a routine gynaecological visit, were selected for cloning of the 16S rRNA genes present in the vaginal microflora. The culture results are reported only for these eight women.

To substantiate the results obtained by cloning and culture, PCR with species specific primers for *A. vaginae* and *G. vaginalis* and culture were carried out for the complete population.

Culture and identification of cultured isolates by tDNA-PCR

For eight non-pregnant women, the swab on Amies transport medium was streaked onto tryptic soy agar supplemented with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ) and incubated anaerobically at 37°C upon arrival at the microbiology laboratory. After 4 days of incubation, all the isolates with different colony morphology were selected for identification. DNA was extracted by simple alkaline lysis: one colony was suspended in 20 μl of 0.25% sodium dodecyl sulfate-0.05 N NaOH, heated at 95°C for 15 min and diluted with 180 μl of distilled water. tDNA-PCR and capillary electrophoresis were carried out as described previously [4,11]. The species to which each isolate belonged was determined by comparing the tDNA-PCR fingerprint obtained from each isolate with a library of tDNA-PCR fingerprints obtained from reference strains, using an in-house software program [11]. The library of tDNA-PCR fingerprints and the software are available on request.

DNA extraction of vaginal swab samples

For DNA extraction from the dry vaginal swabs, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications. The dry swab specimen from each patient was swirled for 15 s in 400 μl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton). Fifty units of mutanolysin (25 U/μl) (Sigma, Bornem, Belgium) were added and the samples were incubated for 30 min at 37°C. After the addition of 20 μl Proteinase K (20 mg/ml) and 200 μl AL buffer (Qiagen), samples were incubated for 30 min at 56°C. Next, 200 μl of ethanol was added and DNA was purified by adding the lysate to the Qiagen columns as described by the manufacturer. Finally, the total bacterial DNA was eluted with 100 μl of AE buffer (Qiagen). DNA-extracts were stored at -20°C and were used for the purpose of cloning experiments and species specific PCR.

Cloning of amplified mixtures of 16S rDNA

To amplify the 5' part of the bacterial 16S rRNA genes by PCR, primers 10f (5' AGTTTGATCCTGGCTCAG) and

534r (5' ATTACCGCGGCTGCTGG) [37], which target the domain *Bacteria*, were used. It should be noted that the forward primer contains mismatches for *G. vaginalis* at positions 1 (A/G), 5 (T/C) and 9 (C/T). A 50 μl PCR mixture contained 0.1 μM of each primer, 25 μl of Promega master mix (Promega, Madison, WI), 5 μl of DNA extract and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 50°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 50°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Cloning was done using the Qiagen PCR Cloning Kit (UA-cloning, Qiagen), whereby the purified amplicons were ligated into the pDrive cloning vector and transformed into Qiagen EZ Competent *E. coli* Cells, as specified by the manufacturer.

Screening for clones with different 16S rRNA gene inserts by ARDRA

From each sample, 100 to 200 ampicillin-resistant transformants, recognizable as white colonies on Luria-Bertani (LB) agar containing IPTG (Roche, Basel, Switzerland), X-Gal (Roche) and 100 μg ampicillin/ml after overnight incubation at 37°C, were selected for further analysis. Single colonies were picked from the agar plates and transferred with sterile tips to the wells of a 96 well plate filled with LB Broth supplemented with 100 μg ampicillin/ml and incubated for 3 h. To avoid sequencing of all clones, the 16S rRNA gene inserts were differentiated initially from each other by means of Amplified rDNA Restriction Analysis (ARDRA)[38]. One μl of bacterial culture was added to a final volume of 20 μl PCR mix, containing 0.2 μM of two plasmid-targeted primers, QF (5'TACGTATCGGATCCAGAATTC) and QR (5'CGAGAAGCTTGTCGACGAATT), 10 μl of Promega master mix and distilled water. The position of primers QF and QR was chosen as such that their distance towards the insert was the same in order to result in the same ARDRA pattern regardless the orientation of the insert in the cloning vector. Cycling conditions were the same as described above for the 16S rRNA gene. Ten microliter of the amplified products were digested with 10 U of the restriction endonuclease *Bst*UI in the appropriate enzyme buffer and incubated for 3 h at 60°C. The DNA restriction fragments were separated in a 2.5% agarose electrophoresis gel, containing 2% Methaphor (FMC Bioproducts, Rockland, ME) and 0.5% MP agarose (Roche, Basel, Switzerland) in the presence of ethidium bromide (50 ng/ml). The gels were photographed and the obtained ARDRA fingerprints were compared visually. For each ARDRA type, the 16S rRNA gene insert of at least one representative clone was sequenced, thus minimizing the number of sequence reactions that had to be carried out.

Sequencing of 16S rRNA genes from isolates and clones

For 8 healthy women, the 16S rRNA gene was amplified and sequenced from selected clones and from cultured isolates that could not be identified by tDNA-PCR, using primers 10f and 534r, as described above. For the clones, 1 μl of the QF-QR amplified PCR product was diluted 1000 times with distilled water and re-amplification of the 16S rRNA gene was done with the primers 10f and 534r. For the cultured isolates, a 5 μl aliquot of the alkaline lysate was added to a 50 μl PCR mixture. The amplification products were then purified with the Qiaquick PCR purification kit, according to the manufacturer's instructions. Sequencing was done using the ABI Big Dye cycle sequencing reaction kit with Ampli*Taq* FS DNA polymerase (Applied Biosystems, Foster City, CA.) with primer 534r. Sequencing reaction products were analyzed on an ABI 310 genetic analyzer (Applied Biosystems). Inspection of the electropherograms was done with Chromas http://www.technelysium.com.au/chromas14x.html and with the BioEdit package [39]. Comparison of the sequences of the inserts and isolates to the 16S rRNA gene sequences in GenBank was done using the BLAST software [40]. Clones or isolates with DNA sequences sharing more than 98% identity with known sequences were assigned to that phylotype.

Species specific PCR for **Gardnerella vaginalis**

G. vaginalis species-specific primers as designed by Zariffard *et al.* (G_Z)[26] and Obata-Yasuoka *et al.* (G_{OY}) [25] were used. Briefly, a 20 μl PCR mixture contained respectively 0.05 and 0.4 μM primers, 10 μl of Promega master mix (Promega, Madison, WI), 2 μl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling with G_Z primers consisted of an initial denaturation of 10 min at 94°C, followed by 50 cycles of 5 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension of 10 min at 72°C. Thermal cycling with the G_{OY} primers was performed by an initial denaturation of 1 min at 94°C was followed by 40 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 70°C and a final extension of 7 min at 72°C. During the first ten cycles the annealing temperature was lowered by 0.5°C per cycle. Five microliter of the amplified product of each PCR was visualized on a 2% agarose gel.

Species specific PCR for **Atopobium vaginae**

Two primer sets that allowed amplification of the 16S rRNA gene of *A. vaginae* and that lacked homology with non-target bacteria as determined by searching the Gene Bank database using BLAST software [40], were designed. The selected primers, ato167f 5' (GCGAATATGGGAAAGCTCCG), ato154f 5' (ATATTTGTCGCATGGCGAAT) and ato587r 5' (GAGCGGATAGGGGTTGAGC), were analysed for secondary structures using NetPrimer (Premier Biosoft Inter-

national, Palo Alto, CA). A 20 μl PCR mixture contained 0.2 μM of primers (respectively ato167f or ato154f and ato587r), 10 μl of Promega master mix (Promega, Madison, WI), 2 μl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 58°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 58°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. Five microliter of the amplified product was visualized on a 2% agarose gel. The primers amplified a DNA-fragment of respectively 420 and 433 basepairs from *A. vaginae* and showed no cross reactivity to other organisms, including *A. rimae* (data not presented).

Statistics

To assess the relative accuracy of PCR-based detection of *G. vaginalis* or *A. vaginae*, we applied comparative receiver-operating-characteristic (ROC) analysis under the non-parametric assumption and compared the accuracies of the isolated markers (amplification with ato154f, ato167f, G_{OY} and G_Z) in allocating subjects to a particular Gram stain category (grades I to IV).

Subsequently, we compared the accuracy of PCR-based combined detection of *G. vaginalis* (using the two different primer sets designated $G_Z$and G_{OY}) and *A. vaginae* (using ato167f) to assess the vaginal microflora status (according to Gram stain category) by non-parametric ROC-analysis.

Accuracy in these analyses is expressed as the area-under-the-curve (AUC) in the ROC-plot, the 95% confidence interval (CI) to the AUC, and the p-value to the 95% CI. We also calculated the estimated sensitivity, specificity, positive and negative predictive values (PPV and NPV) and the 95% confidence intervals to these measures. Statistical significance was accepted at the $\alpha = 0.05$-level.

Analyses were carried out using the statistical software packages EpiCalc2000 v.1.02 and SPSS v.11.0.

Nucleotide sequence accession numbers

Out of 156 16S rRNA gene sequences, obtained from 26 isolates and from 130 clones, two sequences of *A. vaginae* (-like) species and 23 sequences of uncultured bacterium clones were submitted to GenBank and were assigned accession no. AJ585206 to AJ585213 and no. AJ619698 to AJ619714.

Authors' contributions

RV, GC, GV and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. JD participated in the analysis and interpretation of the data and in the writing of the report. LVS and CDG participated in the analysis of the study samples. All authors read and approved the final manuscript.

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