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Development of a species-specific polymerase chain reaction assay for *Gardnerella vaginalis*

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The nucleotide sequence of the region between the 16S and 23S rRNA genes of the facultative anaerobic bacterium *Gardnerella vaginalis* has been determined, together with the 5′ proximal 500 nucleotides of the 23S rRNA gene. Regions suited for the development of specific, probe-confirmable polymerase chain reaction (PCR) assays were selected. PCR assays were evaluated with respect to sensitivity and specificity, the latter in comparison with a number of *G. vaginalis* reference strains and closely related species like *Bifidobacterium* spp. In an initial diagnostic study it appeared that the PCR test detected *G. vaginalis* in 40% of women irrespective of their clinical status. Ten out of 11 patients suffering from bacterial vaginosis as defined on the basis of clinical parameters were carrying *G. vaginalis*.

KEYWORDS: *Gardnerella vaginalis*, polymerase chain reaction, internal transcribed spacer region.

INTRODUCTION

The three main categories of vaginitis are caused by *Trichomonas vaginalis*, yeast or bacteria.[1] Bacterial vaginosis (BV) by itself accounts for up to 50% of all patient cases.[2] It became apparent that a large number of organisms also present in the healthy vaginal flora can become predominant during periods of BV. Among these are species like *Veillonella parvula*,[3] *Mycoplasma hominis*,[4] *Peptostreptococcus* spp.,[5] *Mobiluncus* spp.[6] and *Gardnerella vaginalis*.[7] Most of these organisms have also been implicated as potential causes of BV sequelae like pelvic inflammatory disease,[8] postpartum endometritis[9] and urinary tract infection.[10]

Gardnerella vaginalis is an interesting member of the array of BV-related organisms. An extensive number of microbiological, biochemical and molecular studies has been performed in order to gain insight in the organisms' classification and life cycle.[3,11] *G.*

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vaginalis still is the sole species in the genus and immunochemical detection and identification was described by several authors.[12,13] *G. vaginalis*-specific DNA segments appeared to be useful for species determination[14,15] and a DNA probe specific for *G. vaginalis* rRNA was developed.[16]

In this study we developed a *G. vaginalis*-specific polymerase chain reaction (PCR), which could be used for species identification and detection. The nucleotide sequence of the internal transcribed spacer region as present between 16S and 23S rRNA genes and the 5′ part of the 23S rRNA gene were determined. Based on these sequences several PCR assays were designed and evaluated.

MATERIALS AND METHODS

Bacterial strains and DNA purification

Table 1 surveys the bacterial strains that were obtained from clinical practice or the ATCC, CCUG or LMG culture collections and were grown on agar-based media before DNA extraction. Two subcultures of the type strain of *G. vaginalis* (CCUG 3717^T and ATCC 14018^T) were included. Reference strains were characterized by means of cellular fatty acid analysis[17] and the rapid ID32 strep Microtest (BioMerieux, France) (Falsen, van Esbroeck and Goossens, unpublished data). *G. vaginalis* 2887/10 through 9895/24 are clinical isolates (see Table 1). These isolates were characterised on the basis of colony morphology and a negative catalase test. Special attention was given to *Bifidobacterium* species since 16S rRNA sequence analysis revealed a close relatedness between *Bifidobacteria* and *G. vaginalis*.[18] Cells were harvested in phosphate buffered saline (PBS) pH 7·0 and DNA was isolated and purified by subsequent proteinase K digestion (1 mg ml^{-1} proteinase K in 10 mM Tris-HCl pH 8·0, 1 mM EDTA, 1% SDS for 1 h at 37°C) and phenol extractions. After the extractions DNA was precipitated using ethanol and the resulting pellet was dissolved in distilled water. The DNA concentrations of the final solutions was determined spectrophotometrically and was adjusted to 10 ng µl^{-1}. DNA solutions were stored at −20°C.

PCR amplification

PCRs were performed in Biomed model 60 thermocyclers. The reaction mixtures (100 µl) consisted of 60 mM Tris-HCl pH 9·0, 2·5 mM $MgCl_2$, 50 mM KCl, 0·01% Triton X-100, 0·2 mM desoxyribonucleotide triphosphates, 0·25 U *Tth* DNA polymerase (Sphaero Q, Leiden, The Netherlands) and 50 pmol of both sense and antisense primers. The PCR programme consisted of a 2-min predenaturation step at 94°C. Cycling involved 40 times 1 min 94°C, 1 min 50°C and 1 min 74°C. Reaction products were analysed by electrophoresis through 1–3% agarose gels in 40 mM Tris-acetate pH 7·8, 2 mM EDTA, followed by ethidium-bromide staining and Polaroid photography.

Amplification and sequence determination of the ribosomal intergenic spacer region

In order to determine the internal transcribed spacer (ITS) DNA sequence for *G. vaginalis*, DNA from *G. vaginalis* ATCC 14018^T was PCR amplified using 16S and 23S ribosomal consensus primers (primers #3 and the complement of #5[19]). One of the primers was equipped with a biotin moiety at its 5′ end. After PCR, the biotinylated DNA fragment (approximately 870 bp in length) was bound to Streptavidin-coated magnetic beads (Dynabeads M280, Dynal AS, Oslo, Norway). These beads were subsequently treated with alkali (0·2 M NaOH) and the water phase was neutralized by addition of equimolar amounts of hydrochloric acid and 1/10 volume 1M Tris-HCl pH 8·0. Both the DNA coupled to and eluted from the beads was subjected to manual dideoxy sequencing according to Sanger (T7 sequencing kit, Pharmacia, the Netherlands). ^{35}S-labelled dATP (Amersham, UK) was used for radioactive labelling of the reaction products. Sequence determination was performed by use of the PCR primers and sequencing primers A through F (Table 2).

Selection and evaluation of *G. vaginalis* specific PCR primers and probes

The *G. vaginalis* ITS sequence was compared to similar sequences as determined for *Streptococcus pneumoniae, Anacystis nidulans, Escherichia coli, Rhodobacter sphaeroides, Mycobacterium leprae* and *Streptomyces griseus* using PC gene software and the Genbank sequence depository (Genbank, National Center for Biotechnology Information, Bethesda, USA). Comparisons among this phylogenetically diverse set of prokaryotes led to the determination of various hypervariable DNA sequence loci. Species-specific oligonucleotides (D and G through K) deduced from these regions are indicated in Table 2. Various PCR tests, enabled by the selection of the most variable domains, were performed. The bacteria mentioned in Table 1 were used as a reference panel.

Table 1. Survey of organisms used for the validation of the *G. vaginalis* specific PCR tests

Bacterial species		PCR probe	G/J H	H/K		G/K		
				J	D	H	J	D
Gardnerella vaginalis	CCUG	3717^T	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	3990	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	4123	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	7921	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	11278A	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	28963	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	30587	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	30841	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	7154	+	+	+	+	+	+
Gardnerella vaginalis	CCUG	7712	+	+	+	+	+	+
Gardnerella vaginalis	ATCC	14018^T	+	+	+	+	+	+
Gardnerella vaginalis		2887/10	+	+	+	+	+	+
Gardnerella vaginalis		3064/17	±	±	+	+	+	+
Gardnerella vaginalis		5457/19	+	+	+	+	+	+
Gardnerella vaginalis		5391/18	+	+	+	+	+	+
Gardnerella vaginalis		4841/9	+	+	+	+	+	+
Gardnerella vaginalis		9895/24	+	+	+	+	+	+
Bifidobacterium brevis	LMG	13208	−	−	−	−	−	−
Bifidobacterium longum	LMG	13207	−	−	−	−	−	−
Bifidobacterium magnum	LMG	11590	−	−	−	−	−	−
Bifidobacterium bifidum	LMG	13209	−	−	−	−	−	±
Bifidobacterium infantis	LMG	13203	−	−	−	−	−	−
Lactobacillus acidophilus		01719/8	−	−	−	−	−	−
Lactobacillus acidophilus		01834	−	−	−	±	+	+
Lactobacillus acidophilus		01893	−	−	−	±	+	+
Lactobacillus acidophilus		02028	−	−	−	−	−	−
Lactobacillus acidophilus		01997	−	−	−	±	−	−
Lactobacillus acidophilus		01919	−	−	−	±	+	+
Lactobacillus acidophilus		2604/5	−	−	−	−	−	−
Lactobacillus acidophilus		2604/8	−	−	−	−	−	−
Lactobacillus acidophilus		3313/16	−	−	−	−	−	−
Lactobacillus acidophilus		5656/19	−	−	−	−	−	−
Mycoplasma hominis			−	−	−	−	−	−
Escherichia coli			−	−	−	−	−	−
Chlamydia trachomatis			−	−	−	−	−	−
Bacillus subtilis			−	−	−	−	−	−
Listeria monocytogenes			−	−	−	−	−	−
Streptococcus agalactiae			−	−	−	−	−	−
Shigella flexneri			−	−	−	−	−	−

Gardnerella vaginalis 2887/10 to *Lactobacillus acidophilus* 5658/19 are clinical isolates. For the following organisms the HK PCR in combination with DNA probe D was performed: *Clostridium perfringens, Helicobacter pylori, Enterobacter agglomerans, Campylobacter jejuni, Enterobacter cloacae, Staphylococcus saprophyticus, Klebsiella pneumoniae, Pseudomonas cepacia, Listeria monocytogenes, Citrobacter freundii, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus fragilis, Salmonella serovar godesberg, Haemophilus influenzae, Salmonella serovar senftenberg, Moraxella catarrhalis, Shigella flexneri, Staphylococcus aureus, Bordetella pertussis, Streptococcus agalactiae, Enterococcus faecalis, Cryptococcus neoformans, Staphylococcus epidermidis* and *Candida albicans.*

The PCR conditions were as described above. Amplified DNA was analysed by electrophoresis and after staining with ethidium bromide and Polaroid photography, gels were Southern blotted onto Hybond N^+ nylon filters. Subsequently, the amplified DNA was identified by hybridization with 5'-end-labelled probes. Labelling and hybridization were performed by standard methods.[20] Autoradiography was executed for 3–16 h at −70°C using Kodak X-omat AR films.

Diagnostic application of the *G. vaginalis* PCR test

Twelve CCUG strains of *G. vaginalis* and a single *S. acidominimus* strain of which six displayed abberant biochemical profiles or fatty acid profiles were analysed blindly by the PCR in order to reconfirm the species nature (see Table 3). PCR was performed using primers H and K and amplified DNA was probed with primer D.

Table 2. Survey of oligonucleotides used for sequencing and amplification of the *G. vaginalis* internal transcribed spacer region

Primer code	Aim	Polarity	Position	Nucleotide sequence
A	S	+	512–531 (23S)	GGAGACCCGGCCACTGTTAT
B	S	–	468–449 (23S)	GAGGCATATCGCAGCCCGTC
C	S	–	301–282 (ITS)	GGAGCCGGTTGAAATTGATA
D	S/P	–	669–650 (23S)	CACCTGACGGTTATCACACG
E	S	–	423–404 (23S)	AAGGGCATCCAACCAATACGC
F	S	+	404–423 (23S)	GCGTATTGGTTGGATGCCTT
G	P	+	282–301 (ITS)	TATCAATTTCAACCGGCTCC
H	P	+	381–400 (23S)	TTTACTGGTGTATCACTGTA
I	P	+	536–552 (23S)	GGTACACCACAGTTTTTGTGG
J	P	–	552–536 (23S)	CCACAAAAACTGTGGTGTACC
K	P	–	714–696 (23S)	CCGTCACAGGCTGAACAG

The suffix S in the primer number indicates a sequencing primer, P indicates probe or PCR primer. The *G. vaginalis* specific base sequences were deduced by comparisons with other known ITS and 23S sequences and the location of the primers in either the ITS or the 23S sequence is indicated between brackets.

Table 3. Comparison of biochemical and fatty acid profiling and PCR mediated strain identification for *G. vaginalis*

code nr.	CCUG nr.	fatty acid	ID32 strep	PCR
1	26756	+	+	–
2	4121	+	+	+
3	26920	+	+	+
4	10059	+	+	+
5	7132	–	–	–
6	26996	+	+	+
7	30840	–	+	+
8	28963	+	+	+
9	26921	+	–	+
10	26939	+	+	+
11	7751	+	–	+
12	27435	+	–	±
13	11675	–	–	–

Strain 11675 is *S. acidominimus*. PCR has been performed using primer set H/K combined with probe D. "+" indicates identity, "–" non-identity with the expected value for *G. vaginalis*.

In order to evaluate the clinical applicability of the *G. vaginalis* test and to establish *G. vaginalis* prevalence in women living in the area around or within Delft (the Netherlands), several groups of females were selected. Cervical smears were collected from 52 females attending the gynaecology department for either cervical cytology screening (n=32) or gynaecological problems not related to bacteriological infection (n=20). These patients are considered normal controls, since no apparent growth of *G. vaginalis* was observed upon cultivation of the cervical smears. No clinical signs of bacterial vaginosis were documented either, thereby corroborating the apparent absence of cultivable *G. vaginalis*. Smears were also obtained from 11 women suffering from clinically confirmed bacterial vaginosis (BV). Finally, 20 pregnant females were sampled at time of delivery and the body weight of all babies was determined, as was duration of gestation. Smears were obtained using Ayre spatulas which were immersed in culture medium and transported to the lab, where a DNA isolation was carried out. A 100-µl sample of the smear was incubated with proteinase K and Triton-X100 at 37°C for 1 h. After 10 min of boiling, the material could be used as amplification template.[20] DNA was subjected to two PCRs (GJ and HK primer combination) and reaction products were typed by hybridization with DNA probes. All cervical samples displayed normal cytology and no HPV16 or 18 could be detected by PCR.[20]

RESULTS

Sequence analysis

The nucleotide sequence of a DNA fragment of 855 bp was determined (Genbank accession number L08167). At the 5′ end, 13 bases of the 16S rRNA gene were present, whereas the 23S rRNA sequence started around position 370. This implies that the genuine ITS sequence comprised approximately 360 bp. Using PC gene software, no tRNA genes could be detected within this region. The results of the sequence comparisons, leading to design of the PCR primers and probes mentioned below are available upon request.

Evaluation of PCR assays

The selection of *G. vaginalis*-specific oligonucleotides enabled three PCR tests to be performed. Primer G

could be combined with primer J, making H the probe of choice. Combining primers H and K for PCR makes both primers J and D suitable for probe hybridization. With primers G and K in the PCR, primers H, J and D turn into suitable probes (see also Table 2). The results of PCR/probing analyses are summarized in Table 1. Although some of the non-*Gardnerella* organisms displayed DNA amplification using the specific PCR primers, upon hybridization none of these amplimers crosshybridizes with the probe used (see also Figure 1). Only in case of the G/K PCR some false-positives became apparent. Minor crossreactivity was observed towards *Bifidobacterium bifidum*, but major specificity problems were encountered when *Lactobacillus acidophilus* strains were studied. However, the probes (H, J and D) all appear to be useful, be it in combination with other PCR assays (G/J and H/K). The sensitivity of the assays was in the order of 1 pg of *G. vaginalis* DNA. This should be sufficient for clinical use since in BV *G. vaginalis* is thought to be present in large numbers.

Species typing in relation with biochemical characteristics and fatty acid analysis

Phenotypic analysis of twelve *G. vaginalis* isolates and one isolate of a related species were performed by the ID32 strep Microtest and fatty acid analysis. Generally, the fatty acid content of *G. vaginalis* consisted of 40% palmitate, 33% oleate, 9% myristate, 8% stereate and 3% palmitoleate. A survey of all results obtained is given in Table 3. For two strains (numbers 11 and 12) the identification results of the biochemical and fatty acid analyses were contradictory. Fatty acid analysis identified the strain as *G. vaginalis* which was confirmed by a positive PCR result. Except for strain 1, all remaining strains that are positive in the fatty acid analysis or the biochemical typing or both are identified as *G. vaginalis* by PCR. One strain with abberant fatty acid profile and another strain with a deviating ID32 strep test were still identified as *G. vaginalis* by PCR (isolates 7 and 9, respectively). The results for strain 5, negative biochemistry and abberant fatty acid profile, are corrobarated by a negative PCR result. *S. acidominimus* remains negative in the PCR test.

The identification of *G. vaginalis* by means of phenotypic tests is known to be troublesome and fatty acid analysis often does not distinguish between closely related organisms.[17] However, among this limited number of isolates the results of both pheno- and genotyping assays show a considerable degree of homology. In eight out of 13, all results point in the same direction (isolates 2–6, 8, 10 and 13, see Table 3). For three strains (numbers 9, 11 and 12 in table 3), the ID32 identification is contradictory with PCR and fatty acid analysis. In these cases fatty acid analysis and PCR identify the organism as *G. vaginalis*, whereas the ID32 test is negative in this respect. For one strain (number 7, table 3), fatty acid analysis is negative whereas PCR and ID32 strep testing identify *G. vaginalis*.

Perinatal *G. vaginalis* infections

In the randomized group of gynaecological patients 19 out of 52 women (37%) carried *G. vaginalis*. Ten out of 11 BV patients were positive for *G. vaginalis*. This comprises over 90% of the females in this group. Among the pregnant women, nine out of 20 were colonized with *G. vaginalis* (45%). For a survey of these data, see Table 4. There appears to be no statistically significant difference in prevalence between the control group and the group of pregnant women. Apparently, pregnancy does not predispose to *G. vaginalis* colonization. Children born from *G. vaginalis*-infected females showed an average weight of 3290 g and gestation lasted 39·2 weeks. For children from the non-colonized females the average weight was 3360 g, duration of gestation was 40·1 weeks. This does not constitute a statistically significant difference.

DISCUSSION

A sensitive and specific PCR assay has been developed for *G. vaginalis*. DNA sequences within the internal transcribed spacer and 23S rRNA region appeared to be both species specific and well-enough conserved among isolates to allow adequate test design. For several other species sequence variation has been documented in the ITS region[21] but in *G. vaginalis*, if any variability exists, this does not seem to hamper test results. It has to be mentioned that the most optimal PCR assay (primers H and K combined with probe D) uses sequences from the 23S gene exclusively. In this case potential ITS variability is of no diagnostic concern. Since the PCR discriminates between genuine *G. vaginalis* and *G. vaginalis*-like organisms this test could provide additional clues when identity of a given organism is questionable (see Table 3). A recently published study on *G. vaginalis*-like organisms from the reproductive tract of the mare could profit from additional PCR analysis.[22]

G. vaginalis has repeatedly been indicated as an organism at least partially responsible for various

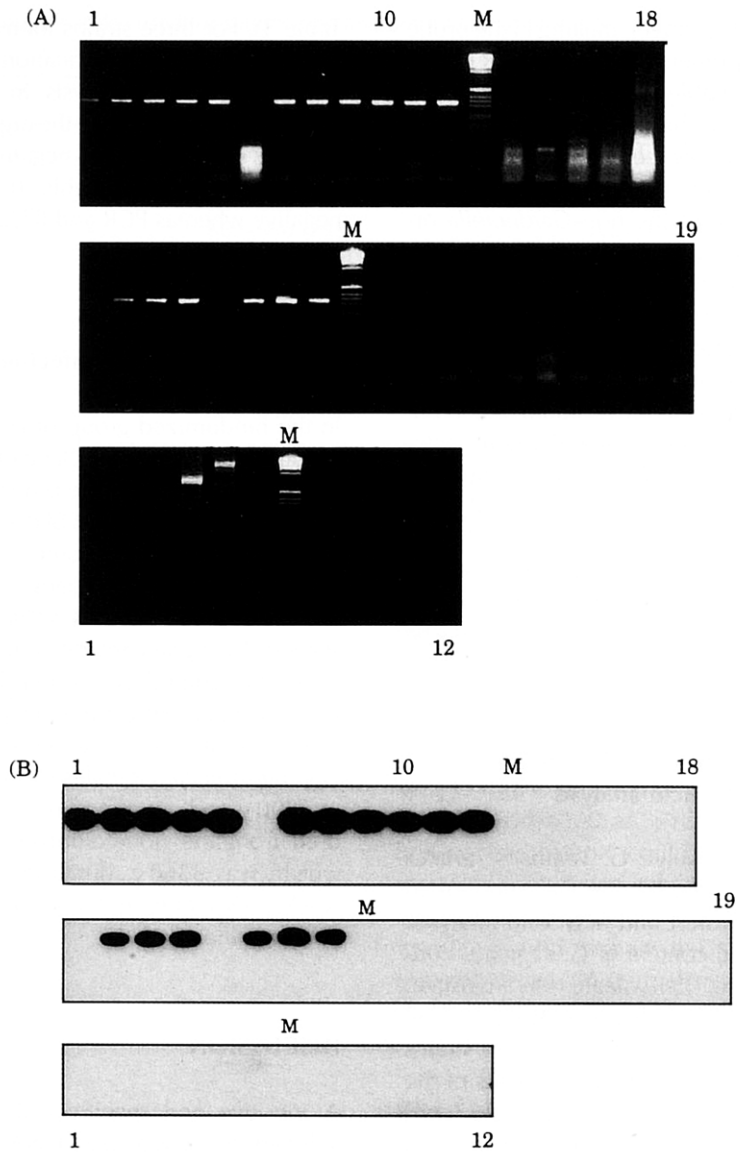

Fig. 1. (A) Survey of amplimers obtained by PCR mediated amplification of DNA from *G. vaginalis*, other micro-organisms and cervical smears by use of primers H and K (see Table 2 for primer sequences). Note that only when *G. vaginalis* DNA is present, is a fragment of the correct length generated, other template DNAs give rise to unspecifically synthesised DNA molecules at best. Lanes marked M contain bacteriophage lambda DNA, digested with the restriction enzyme HindIII, which serves as molecular length marker. The lane numbering corresponds to samples obtained after amplification of DNA from the following materials: *Top panel*, purified DNA from 1. *G. vaginalis* CCUG 3717; 2. *G. vaginalis* CCUG 3990; 3. *G. vaginalis* CCUG 4123; 4. *G. vaginalis* CCUG 7921; 5. *G. vaginalis* CCUG 11278A; 6. *Staphylococcus aureus*; 7. *G. vaginalis* CCUG 28963; 8. *G. vaginalis* CCUG 30587; 9. *G. vaginalis* CCUG 30841; 10. *G. vaginalis* CCUG 7154; 11. *G. vaginalis* CCUG 7712; 12. *G. vaginalis* ATCC 14018/11; 13. molecular length marker; 14. *Bifidobacterium breve* LMG 13208; 15. *Bifidobacterium longum* LMG 13207; 16. *Bifidobacterium magnum* LMG 11590; 17. *Bifidobacterium bifidum* LMG 13209; 18. *Bifidobacterium infantis* LMG 13203; *Middle panel*. DNA from cervical smears from women not presenting symptoms (lanes 1 through 8). Lane 9 displays the molecular length marker, lanes 10 through 19 harbour amplified DNA from the following *Lactobacillus acidophilus* strains: 10. 1719/8; 11. 1834; 12. 1893; 13. 2028; 14. 1997; 15. 1919; 16. 2604/5; 17. 2604/8; 18. 3313/16; 19. 5658/19; *Bottom panel*, amplimers obtained after amplification of 50 ng of purified DNA from the following micro-organisms: 1. *Mycoplasma hominis*; 2. *Escherichia coli*; 3. *Chlamydia trachomatis*; 4. *Bacillus subtilis*; 5. *Listeria monocytogenes*; 6. *Enterococcus* sp.; 7. molecular length marker; 8. *Peptococcus* sp.; 9. *Streptococcus agalactiae*; 10. *Salmonella* serovar godesberg; 11. *Salmonella* serovar flexneri. Lane 12 contains the results of a negative control amplification. Instead of DNA only water was added. (B) Hybridization analysis. DNA from the gels shown in (A) was transferred to nylon and the resulting blot was hybridized with probe D (see Table 2). Note that only DNA synthesised with *G. vaginalis* DNA as template shows specific hybridization with the probe (see also Results and Discussion section).

Table 4. Detection of *Gardnerella vaginalis* by PCR in cervical smears from diverse groups of women

	Present	Absent
Random group ($n=52$)	19/52 (37%)	33/52 (63%)
Pregnant women ($n=20$)	9/20 (45%)	11/20 (55%)
BV patients ($n=11$)	10/11 (91%)	1/11 (9%)

The number of women in each group is indicated by *n*, percentages are given between brackets. BV: bacterial vaginosis.

genital syndromes both in men[23] and women.[24] Multiple studies on neonatal infection have been performed.[25,26] In our patient population, the *G. vaginalis* prevalence in non-symptomatic, non-pregnant females is 36% (19/52 individuals), whereas in pregnant females this percentage amounts to 45%. These percentages exceed the number of carriers (6%) as reported before.[27] However, this latter prevalence was determined by cultivation assays and it may be assumed that the sensitivity of PCR is superior to that of cultivation.

G. vaginalis may be involved in prematurity or growth retardation of the newborn infant. In two previous large studies on the effect of *G. vaginalis* infection on preterm birth, percentages of colonization are low, but both these studies indicate significant differences in degree of colonization in women either giving preterm birth (12% and 23%, respectively) or giving birth at term (6% and 15%, respectively).[28,29] These results are again based on cultivation assays. Data from preliminary studies using PCR as the diagnostic instrument are not yet sufficient to confirm or reject these data (work in progress). It is also reassuring to note that the *G. vaginalis* incidence in patients suffering from bacterial vaginosis is in the order of 90%. Percentages as high as 64–79% and diagnosed by cultivation have been reported.[30]

More extensive studies employing PCR diagnosis of *G. vaginalis* and other BV related micro-organisms are required, whereas also quantification of the actual number of *G. vaginalis* bacteria present may have clinical implications.[31] A technical problem is posed by the fact that PCR, fatty acid analysis and biochemical determination sometimes show contradictory results. If the clinical relevance of *G. vaginalis* infection is to be established, additional evaluation of diagnostic procedures is still required.

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