

CLINICAL STUDY

Complexity of vaginal microflora as analyzed by PCR denaturing gradient gel electrophoresis in a patient with recurrent bacterial vaginosis

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Abstract

Objective: *Gardnerella vaginalis* has long been the most common pathogen associated with bacterial vaginosis (BV). We aimed to test our hypothesis that symptoms and signs of BV do not necessarily indicate colonization by this organism, and often will not respond to standard metronidazole or clindamycin treatment.

Methods: Using a relatively new molecular tool, PCR denaturing gradient gel electrophoresis (DGGE), the vaginal microflora of a woman with recalcitrant signs and symptoms of BV was investigated over a 6-week timeframe.

Results: The vagina was colonized by pathogenic enterobacteriaceae, staphylococci and *Candida albicans*. The detection of the yeast by PCR-DGGE is particularly novel and enhances the ability of this tool to examine the true nature of the vaginal microflora. The patient had not responded to antifungal treatment, antibiotic therapy targeted at anaerobic Gram-negative pathogens such as *Gardnerella*, nor daily oral probiotic intake of *Lactobacillus rhamnosus* GG. The failure to find the GG strain in the vagina indicated it did not reach the site, and the low counts of lactobacilli demonstrated that therapy with this probiotic did not appear to influence the vaginal flora.

Conclusions: BV is not well understood in terms of its causative organisms, and further studies appear warranted using non-culture, molecular methods. Only when the identities of infecting organisms are confirmed can effective therapy be devised. Such therapy may include the use of probiotic lactobacilli, but only using strains which confer a benefit on the vagina of pre- and postmenopausal women.

Key words: PCR-DGGE, bacterial vaginosis, vaginal microflora, diagnosis, probiotics

Introduction

Although bacterial vaginosis (BV) is one of the most common gynecological problems encountered by primary care physicians and gynecologists, diagnostic tools are neither optimal nor convenient. The disease often presents in asymptomatic form, and is detected by the dominance of Gram-negative anaerobic bacteria [1], and in some cases aerobic bacteria [2], replacing the lactobacilli flora found in healthy women. Studies in the last decade have established that BV can be associated with infective complications, such as preterm labor, post-surgical sepsis and an increased risk factor of acquisition of sexually transmitted diseases including HIV [1, 3–5]. The infections frequently recur after antibiotic treatment. Indeed, standard therapy with metronidazole or clindamycin, administered intravaginally or orally,

is followed by relapse in approximately 30% of cases, within 3 months [6]. Arguably, the inability to prevent relapses reflects a lack of understanding of the etiology and microbiology of BV.

The diagnosis of BV is based upon symptoms and signs including vaginal irritation, discharge and fishy odor, followed by microscopic examination of wet mounts. Amsel's criteria [7] or Nugent score [8] determinations, based on microscopic observations of swabs, remain the most widely used tests to diagnose BV. However, these are limited and do not identify the pathogens, which are presumed to be *Gardnerella* and other species such as *Mobiluncus* [9, 10].

Microbiological techniques, such as bacterial cultivation in combination with accurate molecular identification, have aided epidemiological investigation of BV, but they are logistically demanding for routine analysis. In addition, because of anaerobic and

nutritional requirements, a large proportion of vaginal microorganisms are difficult to cultivate and some of them still remain to be cultivated [11]. The use of molecular techniques, based on polymerase chain reaction (PCR) and amplification of 16S rDNA seems to represent a rapid and reliable way to identify microorganisms. The combination of PCR and denaturing gradient gel electrophoresis (DGGE) allows the identification of a high number of bacterial species in the complex ecosystems of the human body [11–13]. DGGE has been successfully used for examination of the vaginal flora, including detection of *Gardnerella* and so far uncultured species such as *Lactobacillus iners* [11, 13]. In the present study, we demonstrate the complexity of the microflora of a woman with recurrent, symptomatic BV which had not been resolved by previous antibiotic or probiotic therapy.

Materials and methods

Subject

A postmenopausal 51-year-old woman (SB) presented with an 18-month history of recurrent BV not resolved by various medications (standard treatment with metronidazole, clarithromycin, antifungals such as fluconazole and antiseptic solutions such as hydrogen peroxide and betadine). She reported having used home-made yogurt and *Lactobacillus rhammosus* GG (10^{10} cells per dose, Culturelle, USA) vaginally and orally, with no detectable improvement with any of the listed regimens. At the time of the study, the patient was not receiving any hormones or antibiotics, but was still ingesting *Lactobacillus rhammosus* GG, one dose daily. Her diagnosis included three of the four Amsel criteria [7], namely vaginal irritation, discharge, elevated pH > 4.5 and presence of clue cells in vaginal swab. She signed an informed consent document approved by the Ethics Review Board at the University of Western Ontario.

Collection procedure

Vaginal swabs were collected by the subject each week for 6 consecutive weeks. The swabs were immediately placed in a transport medium (NCS Diagnostics) and sent to our laboratory for analysis. Smears on microscope slides were Gram-stained and then scored by the method of Nugent et al. [7]: grades 0 to 3 (normal, N), 4 to 6 (intermediate, I) and 7 to 10 (high-grade bacterial vaginosis, BV).

Extraction of DNA

Swabs were vigorously agitated in 1 ml of phosphate-buffered saline (PBS, pH 7.5), and microbial cells

were pelleted by centrifugation (10 000 *g*, 5 min), and washed once in PBS. Microbial DNA was extracted using Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

PCR amplification of DNA and DGGE

Amplicons of ribosomal bacterial DNA were obtained as described previously by Burton and Reid [7]. Briefly, 2 μ l of DNA extracted from the swabs were used as the template of the PCR reaction. The primers used were either eubacterial primers (5'-AC-TCCTACGGGAGGCAGCAG-3', 5'-GTATTAC-CGCGGCTGCTGGCAC-3') in the case of amplification of all ribosomal eubacterial DNA, or lactobacillus-specific primers (5'-AGCAGTAGG-GAATCTTCCA-3', 5'-CATGTGTAGCGGTGR-AAT-3') in the case of the amplification of this specific genus DNA. For each pair of primers, the forward primer carried a GC clamp (40 extra bases, mostly G and C) at its 5' end.

Preparation of DGGE gel gradients and electrophoresis was carried out according to the manufacturer's guidelines for the D-Code™ Universal Detection System of Bio-Rad. A 100 % solution was taken as a mixture of 7 M urea and 40% formamide. The concentrations of polyacrylamide and denaturant were 8% and 30–50%, respectively. PCR products were mixed with 2 \times loading buffer (0.05% bromophenol blue, 0.05 % xylene cyanol, 70% glycerol) and loaded into the wells. Gels were run at 130 V in 1 \times TAE (40 mM Tris, 20 mM acetic acid, 1mM EDTA, pH 8.0). After electrophoresis, gels were stained for 20 min in 5 μ g/ml of ethidium bromide and de-stained for 10 min in 1 \times TAE. Gels were visualized by ultraviolet transillumination.

Band excision from DGGE gels, re-amplification and sequencing

DGGE gel bands were excised using a sterile scalpel, washed once in 1 \times PCR buffer and incubated in of the same buffer overnight at 4°C; 5 μ l of the buffer solution formed the template for PCR amplification. Re-amplification was conducted using either the eubacterial or lactobacilli PCR primers (with no 'GC clamps') depending on the primer set used in the DGGE, and with the same conditions as for the PCR preceding the DGGE analysis. Sequences of the re-amplified fragments were determined by the dideoxy chain termination method (Sequencing Facility, John P. Robarts Research Institute, London, ON, USA). Analysis of the partial 16S rRNA sequences was conducted using the Genbank DNA database and the BLAST algorithm [14]. Identities of isolates were determined on the basis of the highest identity score.

Detection of *Candida albicans* by PCR

Reference strain *Candidia albicans* ATCC 76615 was cultivated on Sabouraud glucose broth or agar (10 g peptone, 15 g glucose and 150 g agar in 1:l distilled water, pH 6.8). The DNA was extracted from washed yeast cells in PBS by using Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The detection of the yeast was based on the amplification of the gene encoding a heat shock protein 90 gene fragment of 317 base pairs [15]. For the detection of *C. albicans* in the DNA extracted from the swabs, 2 µl of template sufficed. The PCR reaction was the same as that described by Crampin and Matthews [15], except that a touchdown PCR protocol was used to increase amplification specificity. The annealing temperature was gradually decreased from 67°C to 55°C for 15 cycles. The detection limit of the yeast PCR assay was determined by using DNA extractions from known numbers of *C. albicans* cells.

Results

Nugent scores

No trichomonads were seen on the vaginal swabs. The six samples tested for Nugent scores showed clue cells and high scores between 9 and 10, confirming high-grade bacterial vaginosis. On microscopy differences were noticed between the six samples, in that the first sample showed a very high proportion of small Gram-negative rods, and in samples from following weeks the proportion of rods decreased as they were replaced by an increasing percentage of Gram-positive cocci.

DGGE analysis of bacterial DNA from vaginal swabs using eubacterial-specific primers

Using DGGE analysis and DNA sequence analysis of the PCR products from eubacterial-specific primers targeting the V2–V3 region of the 16S rRNA gene, it was possible to identify several bacterial species present in the vaginal swabs. Overall, seven different bacterial species were detected: *Klebsiella oxytoca* (fragments 1, 6, 9), *Serratia fonticola* (fragment 2), *Citrobacter freundii* (fragment 3), *Morganella morganii* (fragments 4, 7), *Kluyvera ascorbata* (fragment 5), *Escherichia coli* (fragment 10) and *Staphylococcus epidermis* (fragment 8) (Figure 1). The homology between the sequence obtained from the DGGE bands and the closest species from the database are given in Table I. The DGGE patterns for the first three samples seemed to be quite different to each other, showing an unstable ecosystem. However, for the last 3 weeks of analysis the

DGGE pattern appeared stable. The first sample contained three bacterial species: *Klebsiella oxytoca*, *Serratia fonticola* and *Citrobacter freundii*. On the second week, these three strains were no longer detectable and the microflora was dominated by a *Morganella morganii* strain. This organisms were then detected in all subsequent samples and represented the most intense band overall. Two additional bands, corresponding to *Klebsiella oxytoca* and *Kluyvera ascorbata*, were detected on week 3. Samples from weeks 4 to 6 showed similar patterns with four different bacterial species: *Klebsiella oxytoca*, *Morganella morganii*, *Escherichia coli* and *Staphylococcus epidermis*. These findings correlated with microscopic observations, namely a predominance of Gram-negative rods and Gram-positive cocci.

Interestingly, the sequencing of the DGGE bands did not reveal the presence of *Gardenella vaginalis*, *Mycoplasma hominis*, *Mobiluncus* sp. or *Prevotella* sp. which have generally been associated with cases of BV.

Using eubacterial primers, no DGGE bands corresponded to lactobacillus strains, which are normally present in the healthy vaginal microbiota. This absence of lactobacilli reflected the high Nugent scores of the subject's samples. However, use of *Lactobacillus*-specific primers (Figure 2) did detect five different *Lactobacillus* strains. These comprised *Lactobacillus* sp. GFTH5 (closest species being *L. johnsonii*), *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus delbrueckii* and *Lactobacillus reuteri*. The unidentified lactobacilli were dominant in each weekly sample, whereas *Lactobacillus* sp GFTH5 (*L. johnsonii*), *L. delbrueckii*, *L. johnsonii* and *L. gasseri*

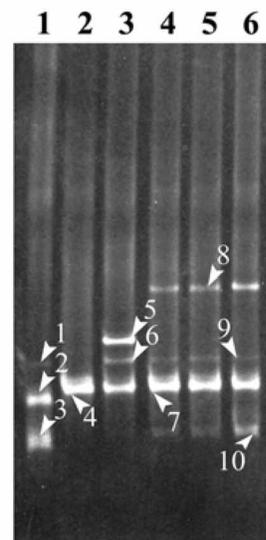


Figure 1. DGGE profiles of the total vaginal microbiota from the subject during the 6 weeks of study (lanes 1 to 6 = weeks 1 to 6). See Table I for bacterial species associated with the arrowed bands shown here.

Table I. BLAST analysis of vaginal bacterial and lactobacilli 16SrRNA sequences of excised fragments from DGGE gels. + : band detected on the DGGE gel, - : band not detected on the DGGE gel, +/- : weak signal on the DGGE gel.

Species	Homology	Band number	W1	W2	W3	W4	W5	W6
<i>Klebsiella oxytoca</i> AF390083	87%	1, 6, 9	+	-	+	+	+	+
<i>Serratia fonticola</i> AF511435	100%	2	+	-	-	-	-	-
<i>Citrobacter freundii</i> CFR233408	98%	3	+	-	-	-	-	-
<i>Morganella morganii</i> AF461011	98%	4, 7	-	+	+	+	+	+
<i>Kluyvera ascorbata</i> AF310219	92%	5	-	-	+	-	-	-
<i>Escherichia coli</i> CFT073 AE016770	98%	10	-	-	-	+	+	+
<i>Staphylococcus epidermidis</i> AF397060DO	92%	8	-	-	-	+	+	+
<i>Lactobacillus</i> sp. GTH5 AF157033	95%	1, 6	+	+	+	+/-	-	+
<i>Lactobacillus johnsonii</i> AY186044	95%	3	+	+	-	-	-	-
<i>Lactobacillus gasseri</i> AY190619	94%	2, 5	+	+	+	-	-	-
<i>Lactobacillus delbrueckii</i> AF375917	92%	4, 8	+	+	-	+/-	+	+
<i>Lactobacillus reuteri</i> LR16SRRI	96%	7	-	-	-	+/-	-	+

W1 = week 1 of sampling etc.

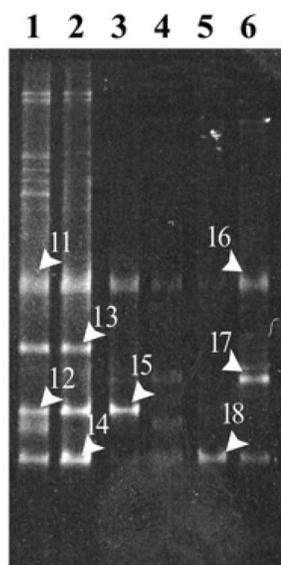


Figure 2. DGGE profiles of the vaginal lactobacilli population from the subject during the 6 weeks of the study (lanes 1 to 6 = weeks 1 to 6). See Table I for *Lactobacillus* species associated with the arrowed bands shown here.

were detected in the second and third weeks only. The DGGE patterns on weeks 4 to 6 were very faint for lactobacilli and only *Lactobacillus reuteri* was identified on week 6. Among the five sequences retrieved from the PCR-DGGE, none of them corresponded to the *Lactobacillus rhamnosus* GG strain present in the probiotic product that the subject was taking orally on a daily basis.

Candida albicans detection

The specificity of the PCR for detection of *C. albicans* was confirmed by DNA sequencing of the PCR products obtained with a *C. albicans* control strain.

The limit of detection was also determined using this PCR method, with a minimum of 100 cells being detected (Figure 3). *Candida albicans* was detected in weeks 1, 2, 4 and 5. The intensity of the PCR fragments from the samples from weeks 1, 2 and 5 was very low, whereas that of fragments from the week 4 sample was intense (between 100 and 1000 cfu/ml, Figure 3).

Discussion

The present study of multiple vaginal samples from a woman with complicated vaginitis, provides valuable insight into difficult cases faced by gynecologists on a regular basis. The bacterial species detected by DGGE using eubacterial PCR primers were all potential urogenital pathogenic species [9, 16, 17]. *Morganella morganii* and *Escherichia coli*, detected in multiple samples, are not generally regarded as specific causes of BV [18], although they are well known for causing UTI [17] and *E. coli* have been associated with aerobic BV [2]. *Staphylococcus epidermidis* was detected in the three last samples from the subject. This skin commensal is not normally a pathogenic species, but can take advantage of immunodeficiency, or abnormalities in the urogenital tract and can cause UTI [17]. Even although the patient was diagnosed with recurrent BV, no *Gardnerella vaginalis* were detected, nor were any of the other species normally associated with this disease, namely *Mobiluncus* sp., *Mycoplasma hominis*, and *Prevotella* sp. [1]. This finding demonstrates the complexity of complicated vaginitis and the difficulties in confirming its diagnosis. It also agrees with a recent study that showed that post-menopausal subjects not receiving estrogen replacement therapy can have BV without *G. vaginalis* [11]. Other studies show that non-gardnerella organisms cause BV [19]

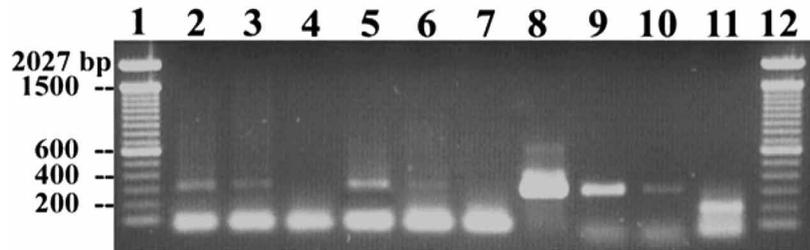


Figure 3. Detection of *Candida albicans* by PCR in the vagina of the subject during the six weeks of the study. Lanes 1 and 12 = 100 bp ladder. Lanes 2 to 7 = weeks 1 to 6. Lanes 8 to 11 = detection of *C. albicans* from a known number of yeast cells (respectively 10000, 1000, 100 and 0 cfu/ml).

and these bacteria can form dense biofilms [20] on vaginal cells, indicative of what is seen on clue cells examined under the microscope.

Given that BV treatment usually comprises antibiotics against anaerobic Gram negative bacteria, several scenarios are possible. Metronidazole treatment may have effectively eradicated infecting anaerobes prior to the patient being entered in our study, in which case the ability of *Staphylococcus epidermis* and *Enterobacteriaceae* to induce symptoms and signs of recurrence of BV is a finding that warrants further investigations. This scenario is less plausible as the subject ceased antibiotic use prior to entering the study. If the patient never had anaerobic pathogens as the prime cause of her BV presentation, then this might explain previous antibiotic failures, and it raises the question of how best to confirm diagnosis of the disease. While it is feasible that *Gardnerella* and other anaerobes may have been present and not detected by PCR-DGGE, it is unlikely over the six week study, and if these organisms were present in such low numbers it is doubtful that they played a major role in the patients' condition. Furthermore, the DGGE method has been shown by us to be a suitable method to detect even low levels of *Gardnerella* [11, 13]. It could be argued that we should have attempted to recover *Gardnerella* by culture, but given the difficulty to recover and quantify anaerobes by culture, and the obvious low numbers present if any, we did not feel that this would have significantly altered the overall findings.

DGGE analysis of eubacterial amplicons did not lead to the detection of any *Lactobacillus* species. However, PCR using species-specific primers did identify lactobacilli in low numbers. This suggests a deficiency in the Nugent scoring system which scores BV based upon the absence of lactobacilli. While these organisms may indeed be 'absent' under microscopy (which generally requires around 10^4 bacteria per ml in the suspension being examined under the microscope), they may in fact not be totally absent from the vaginal vault. This offers hope

for recovery of the patient, as stimulation of the indigenous lactobacilli (through use of prebiotics, or creation of a suitable microenvironment by exogenous lactobacilli application) [21] could lead to recovery and dominance of the lactobacilli flora. Studies have shown that select strains of *Lactobacillus* can colonize the vagina and inhibit the growth and adhesion of various pathogens [22–25]. The failure of *Lactobacillus rhamnosus* GG therapy to lead to its colonization of the vagina and correction of the BV, could have several explanations. Ingestion of this organism has failed in previous studies to make an impact on the urogenital flora and patient health [26, 27], while direct intravaginal application has not led to long term colonization [28]. On the other hand, the patient here is post-menopausal, and in such cases lactobacilli are often not present without estrogen replacement therapy [29], and it is possible that only select probiotic strains would colonize. The fact that lactobacilli were recovered from the vagina of this patient, supports the view that *Lactobacillus rhamnosus* GG [27] is not the most appropriate probiotic for patients with recurrent BV or chronic vaginitis.

Of the lactobacilli detected in the vagina, none were from the yogurt cultures consumed by the patient, and of those cultures only *L. gasseri* has been found to be somewhat common in post-menopausal subjects [11]. The most commonly isolated strain in healthy women after menopause, *L. iners*, was not detected in this subject. Understanding why certain lactobacilli exist in different subjects remains a topic requiring much additional investigation.

PCR proved to be an easy and reliable way to determine the presence of yeast, a common cause of vaginal infections [15, 30]. The extent to which *C. albicans*, contributed to the patient's condition is unknown, but the levels in week 4 were consistent with a transient yeast infection. While the study was not designed to provide a clinical cure for the subject, it is possible that she will require mechanical, chemical and anti-microbial treatment as

reported elsewhere [6] at least a new approach to eradicating the isolated pathogens.

In summary, this study showed that molecular techniques represent an excellent tool to identify the pathogenic microorganisms involved in recurrent BV. Current microscopic wet mount analysis is useful to assess the level of infection, while culture may not be sufficient to adequately guide effective treatment. Until techniques such as PCR-DGGE and sequencing become widely available in diagnostic laboratories, and routine microbiology laboratories more effectively recover fastidious anaerobes from vaginal swabs, physicians will not have adequate information to best diagnose and treat their patients. In this present case, use of metronidazole or clindamycin, the standard agents to treat BV, will only result in failure.

Acknowledgments

We thank Dominique Lam for the staining and reading of slides. This study was part funded by Wyeth Ayerst Canada and NSERC Canada.

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