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Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis

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Abstract

Background: Bacterial vaginosis (BV) is a polymicrobial syndrome characterized by a change in vaginal flora away from predominantly *Lactobacillus* species. The cause of BV is unknown, but the condition has been implicated in diverse medical outcomes. The bacterium *Atopobium vaginae* has been recognized only recently. It is not readily identified by commercial diagnostic kits. Its clinical significance is unknown but it has recently been isolated from a tuboovarian abcess.

Methods: Nucleotide sequencing of PCR amplified 16S rRNA gene segments, that were separated into bands within lanes on polyacrylamide gels by denaturing gradient gel electrophoresis (DGGE), was used to examine bacterial vaginal flora in 46 patients clinically described as having normal (*Lactobacillus* spp. predominant; Nugent score $\leq$ 3) and abnormal flora (Nugent score $\geq$ 4). These women ranged in age from 14 to 48 and 82% were African American.

Results: The DGGE banding patterns of normal and BV-positive patients were recognizably distinct. Those of normal patients contained 1 to 4 bands that were focused in the centre region of the gel lane, while those of BV positive patients contained bands that were not all focused in the center region of the gel lane. More detailed analysis of patterns revealed that bands identified as *Atopobium vaginae* were present in a majority (12/22) of BV positive patients, while corresponding bands were rare (2/24) in normal patients. ($P < 0.001$) Two *A. vaginae* isolates were cultivated from two patients whose DGGE analyses indicated the presence of this organism. Two *A. vaginae* 16S rRNA gene sequences were identified among the clinical isolates. The same two sequences were obtained from DGGE bands of the corresponding vaginal flora. The sequences differed by one nucleotide over the short (~300 bp) segment used for DGGE analysis and migrated to slightly different points in denaturing gradient gels. Both isolates were strict anaerobes and highly metronidazole resistant.

Conclusion: The results suggest that *A. vaginae* may be an important component of the complex bacterial ecology that constitutes abnormal vaginal flora. This organism could play a role in treatment failure if further studies confirm it is consistently metronidozole resistant.

Background

Bacterial vaginosis (BV) is a syndrome that appears to represent a disturbance of the vaginal ecosystem associated with a shift in the microflora [1,2]. Several adverse medical outcomes have been associated with BV [3-11]. While the cause of BV is not understood, some strong associations have been made between the syndrome and the presence of particular bacterial species, such as *Gardnerella vaginalis* and *Prevotella* sp. [2,12]. Most of these, however, represent bacteria that have been detected by cultivation and identified by traditional morphological and biochemical methods.

Cultivation-independent (molecular) analyses, most notably sequencing of 16S rRNA genes PCR-amplified from microbial community DNA, offer a more systematic approach to detecting microbes in natural habitats [13] and a more concrete method of identification and classification [14,15]. Molecular surveys of microbes in environmental samples have repeatedly shown that cultivated species do not represent the full complement of microbes in most habitats [16] and molecular analyses are now commonly used to survey bacterial flora in mammalian systems [17,18].

One technique, denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene segments, offers the possibility of revealing a significant portion of the bacterial flora in a sample as a pattern of bands on an acrylamide gel [19,20]. The bands are separated based on their denaturing characteristics, i.e. sequences and the sequences of individual bands can be obtained allowing the identification of bacteria in the sample. DGGE patterns have been used to reveal changes in microflora and to identify microbes in a variety of habitats [20-26] including human vaginal flora [12,27,28] and other mammalian systems [29-32].

In the course of DGGE analyses of vaginal bacterial flora of normal and BV-positive patients, we detected a band whose sequence matched that of *Atopobium vaginae* (GenBank reference no. AF325325 and American Type Culture Collection [ATCC] #BAA 55) in 55% of BV positive patients. *A. vaginae* is a recently recognized species [33] whose clinical significance is unknown, though it has been identified recently as the cause of a tuboovarian abscess [34]. The isolate described in this case report was found to be highly resistant to metronidazole. We isolated two *A. vaginae* strains from BV patients. Antibiotic susceptibility studies were performed using these two strains and the ATCC #BAA 44 strain. Details of these studies are presented below.

Methods

Subjects

Subjects participated in two different protocols in two locations. In Indianapolis, adolescent females attending one of three adolescent health clinics were enrolled in a prospective cohort study of risk factors for the acquisition of STD's between May, 1999, and September, 2001. They were predominantly African-American (85%) ranging in age from 14 to 17. Having a sexually transmitted disease was not a prerequisite for enrolment but 84% of the cohort were sexually active at the time of testing. Informed consent was obtained from all participants as well as permission from the accompanying parent/guardian for entry into the study. Specimens were collected at the clinics in Indianapolis and were subsequently shipped overnight using cold packs to LSUHSC where they were processed and analyzed.

In New Orleans healthy women between the ages of 18–55 were enrolled into a study of the immune response to *Candida albicans* vaginitis through the Obstetrics and Gynecology Clinic at the Louisiana State University Health Sciences Center (LSUHSC), New Orleans, Louisiana. HIV infected and pregnant women were not eligible for participation in the study. The specimens used in this study were obtained prior to inoculation of *C. albicans*. All clinical research protocols were reviewed and approved by either or both of the Institutional Review Boards at Louisiana State University Health Sciences Centre (LSUHSC), New Orleans, LA, and Indiana University/Purdue University at Indianapolis, Indianapolis, IN.

Specimen collection and processing

Specimens collected from enrolled subjects at both study sites consisted of two vaginal swabs followed by a vaginal lavage. One of the swabs was rolled onto a glass slide, air dried and then Gram stained for the microscopic assessment of BV using the Nugent criteria (0–3: normal, 4–6: intermediate and 7–10: BV) [35]. A vaginal lavage was collected by washing the vaginal vault for 30 to 40 seconds using a syringe and 5 ml of non-pyrogenic sterile saline. One half ml was placed in a sterile vial and frozen at -70°C until thawed for DNA extraction.

Vaginal cultures

A 0.5 ml aliquot of vaginal washes were collected in BBL Port-a-Cul vials from New Orleans women only. The samples were aseptically aspirated from the vial and then plated in a manner allowing quantitation of the most common organisms. Details of the media used and culture methods are available [see Additional file 1]. Colony types present in the highest concentrations were purified and identified using RapID ANA II System (Remel, Inc., Lenexa, KS) and API 20E (bioMerieux, Inc., Durham, NC)

The type *A. vaginae* strain (BAA-55) was obtained from the ATCC and propagated following ATCC instructions.

Susceptibility testing of Atopobium *strains*

A. vaginae strains were tested for susceptibility to various antimicrobials using an NCCLS recommended broth microdilution method for anaerobic bacteria (NCCLS, M11-A5, 2001). Serial two-fold dilutions (0.05–256 μg/ml) of each antimicrobial were prepared in Brucella broth supplemented with vitamin K (1 μg/ml), hemin (5 μg/ml), and laked horse blood (5%). Ampicillin and sulbactam were combined in a 2:1 ratio. The inoculum for each isolate was prepared in pre-reduced Brucella broth by suspending each isolate to turbidity equal to a no. 1 MacFarland and further diluted to give a final inoculum size of 10^5 CFU per well (10^6 CFU/ml). Prior to inoculation all susceptibility plates were pre-reduced in an anaerobic environment for 4–5 hours. Inoculated plates were incubated anaerobically for 48 hours and read. The MIC endpoint was defined as the lowest concentration of each antimicrobial which inhibited the visible growth of the test isolate. Quality assurance was performed using *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741.

DNA isolation

Approximately 0.5 ml of vaginal lavage fluid was used to isolate bacterial DNA. Samples were pelleted in a tabletop micro centrifuge (5 min, 16,000 rpm) and the material was suspended in ~600 μl of phosphate buffer (pH 7.5). Cells were lysed by bead beating and nucleic acids were isolated as previously described [36].

PCR/DGGE/Sequence Analysis

Details of PCR primers were discussed previously [21]. One primer complements a region of the 16S rRNA gene conserved among members of the domain Bacteria (*Escherichia coli* positions 1055 to 1070; 5'-ATGGCTGTCGTCAGCT-3'). The other primer is based on a universally conserved region (*E. coli* positions 1392 to 1406) and incorporates a 40-base GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTGTGTAC-3'). PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension at 72 C for 7 min. All DGGE analyses were performed using a D-Code System along with accessories and reagents essentially as described by the manufacturer (Bio-Rad, Hercules, CA). Gels were 6% acrylamide, gradients were 20 to 80% UF (100% UF denaturant solution is 7 M urea, 40% deionized formamide). Gel spacers were 1.0 mm, glass plates were 16 cm high × 22 cm wide, loading wells were 1 cm wide. Denaturant gradients were formed using a model EP-1 peristaltic pump (Bio-Rad) at a flow rate of 5.0 ml/min and a model GM-20 gradient maker (CBS Scientific, Del Mar, CA). DGGE was run at 60°C at 70 V for 17 hrs. Gels were stained with ethidium bromide and imaged with a Gel-Doc system (Bio-Rad). DGGE bands were purified for sequencing by repeated transfer, PCR amplification and DGGE analysis as previously described [36].

Sequencing was performed by the LSUHSC genomics core facility. Raw sequence data were aligned and edited using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI) and compared to sequences in public databases using BLAST [37].

Results

DGGE banding patterns of normal and BV positive samples

Lavage samples were obtained from 24 patients with normal vaginal secretion Gram stains, from two patients with intermediate vaginal flora and from 20 with BV based on Nugent's score. Two distinct types of banding patterns were observed. The first, designated the normal type, contained from 1 to 4 bands that were concentrated in the centre region of the gradient gels (Fig. 1B). The sequences of these bands were consistently those of *Lactobacillus* spp., mainly *L. crispatus* and *L. jensenii*. When a patient's vaginal flora was normal by Nugent's score, their DGGE pattern was the normal type in 92% (22/24) of cases. A second commonly observed DGGE pattern was designated the BV type, unlike the normal type these patterns contained bands that were not all focused in the center region of the gel and often contained more than 4 bands (Fig. 1A). In addition to *A. vaginae* (see below) sequences from bands in the BV gels included *Gardnerella vaginalis*, *Bifidobacterium* spp. *Mycoplasma* sp. *Prevotella* sp. and *Lactobacillus* sp. We did not sequence all bands in this study. In all 22 patients with either intermediate vaginal flora or BV by Nugent's Gram stain score, their DGGE pattern was the BV type.

Examples of DGGE banding patterns obtained from 16S rRNA gene segments PCR-amplified from nucleic acids isolated from vaginal lavage samples of patients clinically described as having BV-positive (A) or normal (B) vaginal flora. The boxed area encloses examples of *A. vaginae* bands. These were found in 55% of BV-positive samples.

Identification of A. vaginae *by DGGE*

We observed that two DGGE bands which migrated to nearly the same position in the lower region of DGGE gels were common in vaginal samples of BV-positive patients but were not common in normal patients (Fig. 1 and 2, boxed areas). Of the 14 patients whose DGGE patterns contained these bands, seven contained both bands. The boxed areas in Figure 2 provide an example of the double band pattern. (Fig. 2A lane 1 and 2B lane 1). The 16S rRNA sequences of two of the upper bands and five of the lower bands were determined; all were *A. vaginae*. The

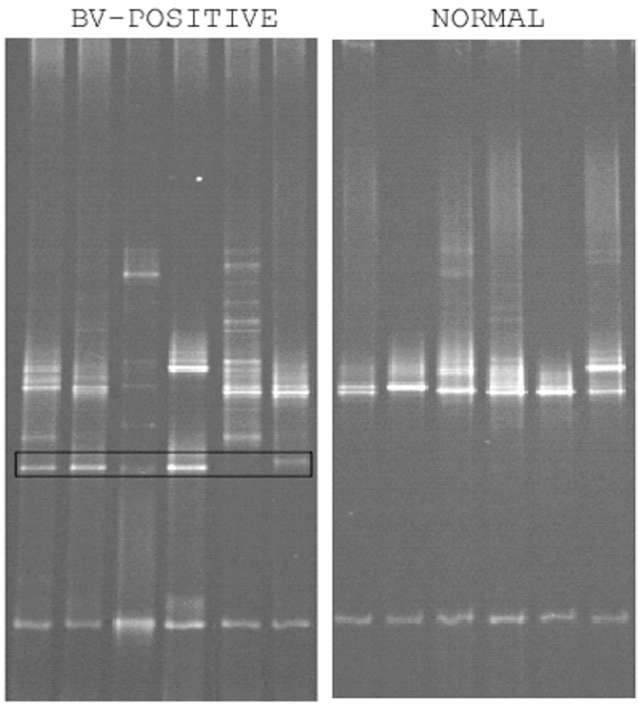

Figure 1
Normal vs. BV positive DGGE banding patterns
Examples of DGGE banding patterns obtained from 16S rRNA gene segments PCR-amplified from nucleic acids isolated from vaginal lavage samples of patients clinically described as having BV-positive (A) or normal (B) vaginal flora. The boxed area encloses examples of *A. vaginae* bands. These were found in 55% of BV-positive samples.

sequences of bands that migrated to the slightly higher position in the gradient gel (for example Fig. 2A, lane 2 and Fig. 2B, lane 3) were exactly the same as that of *A. vaginae* (GenBank reference no. AF 325325) while bands that migrated to the slightly lower position (for example Fig. 2A lane 3 and Fig. 2B lane 2) differed from *A. vaginae* AF325325 by one nucleotide (a G instead of an A at nucleotide 1220 of *A. vaginae* GenBank reference AF325325).

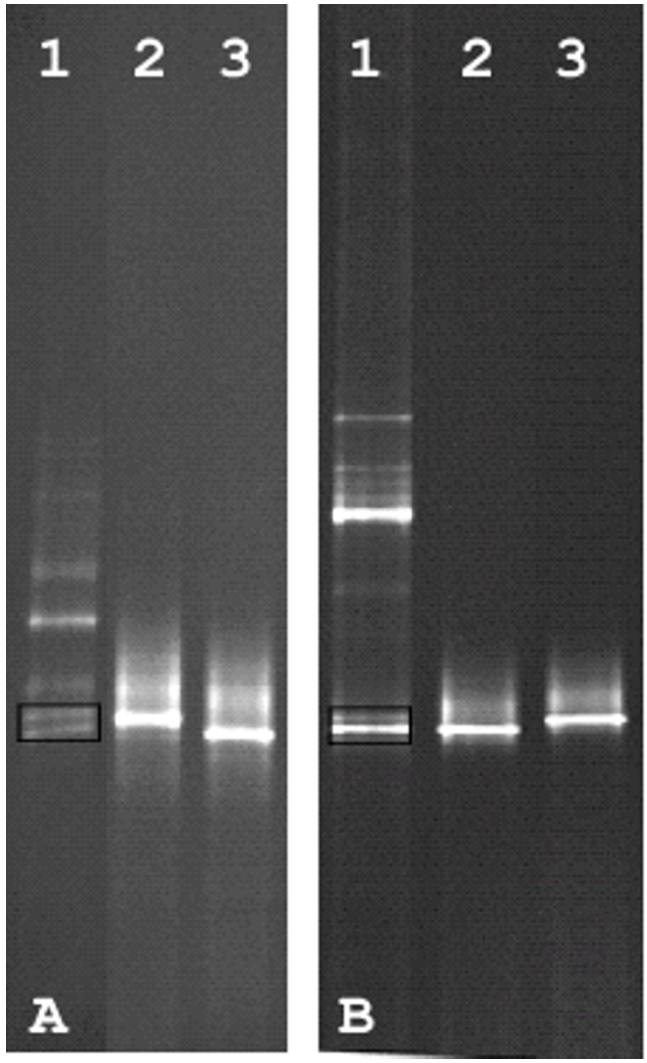

Figure 2
Identification of two *A. vaginae* strains by DGGE using PCR amplified segments of the 16S rRNA gene
Panel A, lane 1 is from vaginal lavage sample #7189A obtained from a patient with BV. Note the closely opposed *A. vaginae* bands in the boxed area. Lanes 2 and 3 show the individual *A. vaginae* bands from the same sample after extraction and re-amplification of the two bands in lane 1. Panel B, lane 1 is from a vaginal lavage sample #7200A obtained from another BV positive patient #7200. It also shows the double *A. vaginae* band pattern (boxed area). Lane 2 shows the band obtained from an *A. vaginae* strain isolated from patient #7200. This band co-migrates with the lower of the two bands obtained from this patient's vaginal lavage sample. Lane 3 shows the band obtained from the ATCC *A. vaginae* culture collection strain. It co-migrates with the upper of the two *A. vaginae* bands shown in lane 1. The slight difference in the electrophoretic migration of the *A. vaginae* bands correlates with a single nucleotide difference in their amplicons.

Table 1: Antibiotic susceptibility. MIC values (μg/ml) determined by NCCLS broth microdilution of *Atopobium* isolates to various antimicrobial agents.

	ATCC BAA55	DM7193	DM7200
Metronidazole	>32	>32	>32
Clindamycin	<0.015	<0.015	<0.015
Ceftriaxone	2	0.5	.5
Cefoxitin	2	2	2
Ampicillin/sulb	0.25	0.25	.06
Meropenem	0.5	0.03	<0.015
Imipenem	0.5	0.03	<0.015
Moxifloxacin	0.12	0.06	1
Trovafloxacin	0.03	<0.015	2
Linezolid	0.25	.25	.06
Disk Tests			
Aminoglycosides*	R	R	R
Vancomycin*	S	S	S

*done by disk diffusion as a screening test

Although the segment of the 16S rRNA gene used in our DGGE analysis is only ~300 nucleotides, it includes a highly variable region of the molecule [21] and the sequences did not match other (non-*A. vaginae*) 16S rRNA sequences in BLAST analyses [37]. Based on the presence of bands at the appropriate position in denaturing gradient gels (illustrated by the boxed area in Figures 1 and 2), 55% (12/20) of BV positive samples contained *A. vaginae*, as opposed to 8.3% (2/24) of normal samples ($P < 0.001$ by Chi Square). In the two cases where *A. vaginae* bands were present in patients with "normal" vaginal secretions, both exhibited complex (BV-like) DGGE patterns (data not shown).

A. vaginae culture identification and antibiotic susceptibility

The *A. vaginae* ATTC strain (GenBank reference No. AF325325) isolated from a women with a tubo-ovarian abscess was reported to have coded out as *Gemella morbillorum* using the API ID32A system. [34]. We reviewed our vaginal culture results and found two cases (7193 and 7200) in which cultivated isolates were identified as *G. morbillorum* using the RapID ANA II system. The vaginal lavage fluid DGGE pattern from these two patients contained bands identified as *A. vaginae*. These bacteria grew only under anaerobic conditions as tiny grayish non-hemolytic colonies. Gram stains revealed small Gram positive cocco-bacilli. These two isolates were passed to assure purity and then subjected to DNA extraction, PCR-amplification and DGGE analysis. The strain from patient 7200 contained a single dominant band that corresponded to the lower of the two *A. vaginae* bands observed in BV-positive patients (Fig. 2B lane 2). Despite having been grown from a single colony, the strain from patient 7193 contained both *A. vaginae* bands in its DGGE pattern (data not shown). Sequencing confirmed that one of these bands was identical to the GenBank *A. vaginae* AF 325325 sequence while the other had the single nucleotide change described above. It may be that the 7193 strain is not axenic, alternatively, the strain may contain both copies of the 16S rRNA gene [38-43]. Antibiotic susceptibility of our two *A. vaginae* isolates and the ATCC type strain are summarized in Table 1. Though all three organisms were susceptible to most of the antibiotics tested they were highly resistant to metronidazole.

Discussion

The genus *Atopobium* was proposed in 1992 [44] to accommodate bacterial isolates previously classified as *Lactobacillus minitus, L. rimae* and *Streptococcus parvulus*. The species *A. vaginae* (GenBank accession no. Y17195) was defined in 1999 from a vaginal isolate of a "healthy patient" though no description of the nature of vaginal flora in this patient was given [33]. More recently, *A. vaginae* (GenBank accession no. AF325325) was reported as the infective agent in a tuboovarian abscess [34]. The 16S rRNA gene sequences of Y17195 and AF32325 are indistinguishable though there are four undefined nucleotides in the sequence of the former.

This is the first report of the association of *A. vaginae* with abnormal vaginal flora. The appearance of prominent *A. vaginae* bands in 55% of patients with Nugent scores >3 suggests that the species was prominent in patients with abnormal vaginal secretions. The presence of *A. vaginae* DGGE bands in only 8.3% of samples from patients with normal flora suggests that *A. vaginae* was not prominent in normal vaginal secretions. It is unlikely that the frequency of the *A. vaginae* bands in BV-positive samples, and their absence from normal samples, is an artifact of the PCR amplification/DGGE method. The PCR primers complement sites on both *Lactobacillus* spp. and *A. vaginae* 16S rRNA genes, and *A. vaginae* and *Lactobacillus* bands co-occur in some specimens (Fig. 1). Thus there is no evi-

dence that primer bias toward *Lactobacillus spp*. would explain the lack of *A. vaginae* bands in DGGE patterns of normal patients. The appearance of prominent *A. vaginae* bands in DGGE patterns of BV-positive patients is likely a true reflection of their prevalence in the bacterial ecology that constitutes the BV syndrome.

In some cases complex (BV-like) DGGE patterns were obtained from patients with normal vaginal secretion Gram stains. In fact this was the case in both samples that were normal by Nugent's criteria but also produced *A. vaginae* bands by DGGE analysis. There are almost certainly instances when vaginal flora cannot adequately be classified as normal or BV positive using the current criteria [45]. We noted an array of DGGE patterns among patients diagnosed with BV. (Fig. 1A). These PCR-generated patterns provided a more objective and informative assessment of vaginal flora than standard clinical and/or Gram stain criteria. The ability to more fully resolve vaginal flora into patterns using DGGE, combined with the ability to correlate these patterns with bacterial species and aspects of disease, such as onset, progression or response to antibiotics, could lead to a better understanding of the BV syndrome [12,27,28].

As part of our evaluation of the potential role of DGGE in assessing vaginal flora we had evaluated a series of patients using quantitative culture techniques. The details of this evaluation will be the subject of a future publication. After detecting *A. vaginae* using molecular techniques, we reviewed our culture results to determine if any of our isolates had been identified as *G. morbillorum*, based on the report by Geissdorfer et al. [34] that their *A. vaginae* isolate was originally misclassified as this organism by the API ID32A system. We found that two of our isolates had been identified as *G morbillorum* using the RapID ANA II system. As did Geissdorfer et al., we found that our isolates were actually *A. vaginae* based on 16S rRNA gene sequencing and both proved to be strict anaerobes as was theirs. Of note is the fact that despite the original description of *A. vaginae* as a facultative anaerobe by Rodriquez Jovita et al [33] we found it to be a strict anaerobe using the type strain submitted to ATCC by these investigators. The reason for this discrepancy is unclear but the weight of evidence thus far indicates that *A. vaginae* is a strict anaerobe.

It is not surprising that *A. vaginae* has not been previously recognized in women with BV. The Gram stain appearance is that of a small Gram positive cocco-bacillus and members of the genus are known to produce large amounts of lactic acid. On this basis, some species belonging to the genus *Atopobium* were originally identified as *Lactobacillus sp*. In fact it was in the course of a study of vaginal lactobacilli that *A. vaginae* was originally identified [33]. To our knowledge *G morbillorum* has not been reported in previous descriptions of normal or abnormal vaginal flora. Differences between bacteriologic methods could explain this discrepancy. It may be that some identification systems do not correctly separate anaerobic lactobacilli and streptococci from *A. vaginae*.

A potentially important observation is that *A. vaginae* appears to be highly metronidazole resistant. We have extended the observation of Geissdorfer et al [34] who first described metronidazole resistance in this species using their strain. Our two isolates and the originally described type strain are highly metronidazole resistant. Therefore, as all four known *A. vaginae* isolates are metronidazole resistant, this may be a characteristic of this species. Additional testing of new isolates will be required to confirm this possibility. Geissdorfer et al [34] reported their strain to be susceptible to penicillin, cefuroxime, cefoxitin and impenem. We found the organisms to be susceptible to clindamycin, cephalosporins, carbapenems, ampicillin/sulbactam and linezolid and were moderately susceptible to the quinolones. (Table 1). By disc diffusion our strains were resistant to gentamicin but susceptible to vancomycin. It is interesting to speculate that this organism could possibly contribute to BV treatment failures or relapses which are common [2]. Prospective treatment studies are needed to determine whether or not *A. vaginae* and other metronidazole resistant organisms such as *Mobiluncus curtisii* might play such a role.

Conclusions

DGGE ananysis of segments of PCR-amplified 16S rRNA gene sequences appears to be a useful tool in describing the complex bacterial flora associated with BV, and relatively simple flora associated with normal vaginal environments. Our observations require confirmation in other populations of women, but it appears that *A. vaginae* may be a common component of the microbial flora associated with BV. *A. vaginae* would likely not be effectively cleared using metronidazole, the most common antibiotic used to treat BV. Further prospective treatment studies should be undertaken to elucidate the possible significance of *A. vaginae* in BV treatment failures or relapses.

Competing interests

None declared.

Authors' contributions

The manuscript was written by MJF and DHM. MJF and AM contributed DGGE analyses and 16S rDNA-based bacterial identification. DHM supervised the microbiology laboratory work and KEA supervised the antibiotic susceptibility testing. JDF and PLF provided clinical samples, patient data. KEA, JDF and PLF critically reviewed the manuscript.

Additional material

Additional File 1

Detailed description of culture methods. This file provides additional details of methods used to cultivate organisms, including those not presented in the manuscript.

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