Organisms associated with bacterial vaginosis in Nigerian women as determined by PCR-DGGE and 16S rRNA gene sequence

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Abstract

Background: Bacterial vaginosis (BV) is a condition with diverse etiology. This condition predisposes women to increased susceptibility to sexually transmitted diseases, including human immunodeficiency virus (HIV) infections and preterm birth. The diagnostic methods currently adopted in the evaluation of patient samples for BV are arguably Amsel criteria, and Nugent score that require microscopy and expert interpretation. These two methods are still subjective.

Objective: The objective of this study was to determine the organisms present in the vagina of 34 HIV negative Nigerian women diagnosed as having bacterial vaginosis by using molecular techniques.

Methods: The vaginal samples were subjected to DNA extraction, and amplified with eubacterial primers via PCR. The PCR products were separated using denaturing gradient gel electrophoresis (DGGE). Bands were excised, re-amplified, purified and sequenced. Sequence identification was performed using the BLAST algorithm and Genbank data base.

Results: *Mycoplasma hominis* (12/34; 35%) was the most common isolate and 9 (26%) contained one of two clones of an unusual Rainbow Trout intestinal bacterium, while unculturable *Streptococcus sp*, and other bacteria made up the remaining isolates.

Conclusions: The findings indicate further diversity in the etiological agents associated with BV, and raise the question as to whether diagnosis and management of this condition needs to be re-evaluated in countries like Nigeria. There is some controversy over the clinical importance of BV, as it was once regarded as a disease caused by *Gardnerella* and presenting as an odourous discharge condition, but is now diagnosed without necessarily the presence of these organisms or signs. With the incidence of BV aligned to an increased risk of HIV in a country ravaged by this virus, the effective eradication of BV can only be achieved if appropriate therapies are delivered.

Keywords: bacterial vaginosis, Nigerian women, mycoplasma

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Introduction

Bacterial vaginosis (BV) is a condition characterized by a shift in the vaginal microbiota from a predominant lactobacilli population to one dominated by anaerobic *Gardnerella vaginalis, Prevotella, Bacteroides* and *Mobiluncus* species [1]. A condition termed aerobic vaginitis has been coined for a smaller proportion of women whose microbiota is dominated by streptococci and *E. coli* [2]. BV is estimated to affect more than 10% of the female population world-wide [3], but prevalence varies from 30% in Indonesia, 25% in Canada [4,5] to15% in rural Brasil [6]. A recent study by our group showed a rate of 14.2% for BV amongst a population of Nigerian women [7]. The aim of the present study was to determine the microbial contents in these subjects. A gene-based, rather than culture-based, method was used as the latter fails to detect many of the organisms that inhabit the vaginal tract [8]. Indeed, we have previously demonstrated in the vagina the high prevalence of *Lactobacillus iners*, an organism that does not grow on standard lactobacilli media, and *Atopobium vaginae*, an anaerobe associated with cases of BV [9,10]. BV is diagnosed clinically in several ways, depending upon availability of methods, cost and experience of clinicians. The Amsel criteria is based upon microscopic examination of vaginal discharge to identify "clue cells", vaginal cells covered in Gram negative

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bacteria, and two of the three following criteria: (a) anterior fornix vaginal pH >4.5, (b) release of a fishy odor on addition of 10% KOH to the vaginal discharge (positive "whiff" test), and (c) presence of an increased thin homogeneous white vaginal discharge [11]. A Gram stain method known as the Nugent Score, is also used at some centres [12]. It is based on a scoring system ranging from normal (predominantly lactobacilli rods) to BV (absence of lactobacilli and domination by Gram negative rods and Gram positive coccobacilli). In Nigeria, the Gram stain method is often used. The diagnosis of BV leads to antibiotic administration primarily using metronidazole, or as a second option clindamycin. With increasing drug resistance [13], the choice of empirical therapy may increasingly require first the proper identification of the causative agents.

Materials & methods

Study subjects

Thirty four asymptomatic premenopausal women (18-48 years) whose Nugent scores (7-10) were interpreted as bacterial vaginosis (BV) with absence of Gram positive rods (lactobacilli), and presence of small Gram negative or Gram variable rods were enrolled. In order to confirm the BV status of the patients, a commercially available test kit known as BV Blue was used. This test is based on the fact that sialidase is produced by most BV organisms [14] .The swabs were collected from the posterior fornix of the vaginal tract from 34 women with BV, packaged and placed in ice packs and transported by courier to the Lawson Health Research Institute, London, Canada, for bacterial DNA extraction and sequencing. The Ethics committee of the Faculty of Pharmacy, University of Benin approved the study.

Extraction of bacterial DNA from Vaginal Swabs

Bacterial DNA was extracted from the vaginal swabs using Instagene Matrix (Bio-Rad Laboratories) according to manufacturer's instructions. Briefly, swabs were vigorously agitated in 1mL of PBS (phosphate buffered saline, pH 7.1) to dislodge cells. The cells were pelleted by centrifugation (Eppendorf, Digital Centrifuge 5417C) at 10,000g for 5 minutes, washed by re-suspending cells in PBS and centrifuged at 13000g for 3 minutes. The pellets were resuspended in 200µl Instagene Matrix, incubated for 20-30 minutes in a water bath (Isotemp®, Fisher Scientific, USA) at 55°C. The sample was vortexed for 10 seconds and boiled at 100°C (Tekstir® Hot plate) for 8 minutes. The sample was vortexed for 10 seconds and centrifuged at 13,000g for 3 minutes. The supernatant containing the DNA was stored at –20°C.

PCR amplification of the DNA Template/Sample

The amplification reactions of the DNA template/sample were carried out in single 0.2mL PCR tubes (RNase/DNase/pyrogen free [Diamed, lab Supplies, Mississauga, ON, Canada]) using a Thermocycler (Eppendorf Mastercycler). Each PCR consisted of 5.0µL of 10Xbuffer (No MgCl2), 10mM Tris-HCl, and 50mM KCl), 2.5µL of MgCl2 (50mM), 1.0µL dNTPs (5mM each), 1.25µL of glycerol (80%) (Sigma), 4.0µL of Bovine serum albumin (BSA) (10mg/ml) (Sigma), 50 pmoles/µL of each primer HDA-1 GC (Primer length 60) with the Sequence (5' to 3'); CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GACTCCTAC GGG AGG CAG CAG, and HDA-2 (Primer length 21) with the sequence(5'to 3'); GTA TTA CCG CGG CTG CTG GCA, (Invitrogen™, Life Technologies), 0.2µL of Platinum® Taq DNA polymerase (5U/µL) (Invitrogen™, Life Technologies), 2.0µL of the DNA template/sample, and sterile water (Fluka H_2O) to a volume of 50µL. PCR amplification involved an initial DNA denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and elongation at 72°C for 1 min, which was followed by a final extension at 72°C for 10 min. To confirm amplicon production, the mixture (5µL PCR product and 2µL of loading buffer) was analyzed by electrophoresis (Bio-Rad) using 1.5% Ultrapure™ Agarose (Invitrogen, Life Technologies) gels at 100 volts for 45 min, followed by staining with 1% solution of ethidium bromide (50µl/L) and de-staining with 1xTAE for 10 min. Gels were visualized by UV transillumination and recorded with Polaroid 667 instant film.

DGGE (Denaturing Gradient Gel Electrophoresis)

In DGGE, the denaturing environment is created by a combination of uniform temperature, typically between 50 and 65°C and a linear denaturant gradient formed with urea and formamide. Preparation of gel gradients and electrophoresis was carried out according to the manufacturer's instructions for the D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories). A solution of 100% chemical denaturant consists of 7 M urea and 40% formamide. The concentrations of the polyacrylamide, denaturant and Tris-acetate buffer (40mM Tris base, 20mM glacial acetic acid, 1mM EDTA [pH 8.0]; TAE) were 8%, 30%-50%, and 50x, respectively. Solutions were degassed for 10-15 minutes and filtered through a 0.45µM pore size membrane (Nalgene, USA) using a vacuum pump. 500µL of D-Code Dye was added to the 50% denaturant solution. Polymerization agents (55µL of TEMED- [N,N,N'N'-

tetramethylenediamine;Sigma] and 95μL of 10% ammonium persulphate [Bio-Rad] were added. Prepared gel concentrations were cast on a parallel gradient gel stand using the Model 475 gradient delivery system. Gels were allowed to polymerize overnight. 20μL of amplified PCR products were mixed with 20μL of 2x loading buffer (0.25mL of bromophenol blue [2%, Sigma], 0.25mL of xylene cyanol [2%, Sigma], 7mL of glycerol, and 2.5mL of distilled H2O) and loaded into the wells. Gels were run at 130 Volts for 3.5-4 hours in 1x TAE until the second dye front (xylene cyanol) approached the end of the gel. After electrophoresis, gels were removed and allowed to cool under flowing tap water before the removal of the glass-plate sandwich. The gel was stained for 15 minutes in 5μg/mL of ethidium bromide, and de-stained for 10 minutes in 1x TAE. Gels were visualized by UV transillumination, and the results were recorded using polaroid 667 instant film.

Band excision from denaturing gradient gels

DGGE fragment bands were excised from the gels with a sterile scalpel and placed into single 1mL Eppendorf tubes. Cut bands were washed in 1xPCR buffer and incubated in 35μL of the same buffer overnight at 4°C.

PCR Re-amplification of the cut bands

Five microlitres of the buffer solution containing fragments from the previously excised bands was used as template for PCR re-amplification. This was conducted using the same PCR Master Mix to a total volume of 50μL and eubacterial primers HDA-2 and HDA-1 without the GC clamp. The amplification, annealing and extension conditions were the same as described above.

Purification of the re-amplified PCR product

The double stranded DNA fragments from PCR reactions were purified using QIAquick Purification Kit protocol (QIAGEN Inc. Mississauga, Ontario, Canada). Briefly, 5 volumes of Buffer PB was added to 1 volume of the PCR re-amplified sample and mixed in a microcentrifuge tube. This was placed in a 2mL collection QIAquick spin column and centrifuged for 60 sec. The flow-through was discarded and the DNA content in the column was washed with 0.75mL buffer PE and centrifuged for 60 sec twice. The flow-through was discarded and the spin column was placed in a clean 1.5mL microcentrifuge tube. The DNA was eluted by adding 30μL fluka sterile H2O at the center of the QIAquick membrane, left to stand for 1-20 min and centrifuged. The 30μL flow-through contained the purified DNA.

Sequencing

Four μL of the purified DNA mixture was added to 4μL of Big-Dye Terminator Reaction Mix (Applied Biosystems, USA), plus 1.6μL of primer and 0.4μL of de-mineralized H2O. The sequencing reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer, USA) with 30 cycles of 96°C for 20 s, 50°C for 15 s, and 60°C for 4 min. The temperature was lowered to 4°C after the last cycle. The sequencing products were purified through a column comprised of G-50 Sephadex (Sigma, St. Louis, USA), dried in a Speed-Vacufuge SVC100 (Savant, Instruments Inc, New York) for 1 hour and re-suspended in a loading buffer (10μL formamide). Sequences of the fragments, were determined by the automatic Big Dye (dideoxy chain terminator) sequencer ABI PRISM 3730, (Sequencing Facility, John P. Robarts Research Institute, London, Ontario). Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2.3 (Chromas version 2.3; www.technelysium.com.au.chromas.html). For identification of the closest relatives, newly determined sequences were compared to those available in the V2-V3 region of the 16S rRNA sequences using the GenBank DNA databases (www.ncbi.nih.gov) and the standard nucleotide-nucleotide BLAST algorithm [15]. The identities of the sequences were determined on the basis of the highest percentage of total nucleotide match in GenBank.

Results

Of the 34 samples, 10 (29.5%) had four bands, 16 (47.0%) had three bands, 6 (17.6%) had two bands and 2 (5.9%) had one band. Based upon the strength of the banding patterns, the dominant organism in 35% of the samples was *Mycoplasma hominis* (Table 1) as revealed by the 16S rRNA gene sequence and analysis using the Basic Local Alignment Search Tool (BLAST) in the Genbank data base. *Mycoplasma hominis* with AJ002269 as gene bank accession number had 98% identity to the sequences deposited in the gene bank database of the National Centre for Biotechnological Information (NCBI). The other dominant bands comprised uncultured *Streptococcus* sp having 98% identity, and AJ307987 as gene bank accession number, with 842 DNA base pair sequence length and in 8 (24%) of the vaginal samples. An uncultured bacterial clone HuCB85 (15%) was present in 5 (15%) of the BV-scored vaginal samples. The uncultured bacterium had 97% similarity with AJ409007 as gene bank accession number and 1513 DNA base pair sequence length. An unusual organism matching a Rainbow trout intestinal bacterium with two clones T1 and D22 was identified in nine (26%) of the

Table 1: Summary of organisms identified by Gene Bank BLAST algorithm.

Number of samples	Percentage (%)	Bacteria identified by 16S rRNA Gene sequence	Gene Bank Accession Number	% Identity	DNA base pair sequence length
12	35	*Mycoplasma hominis*, rrnB operon, strain 7488	AJ002269	96	1857
9	26	Rainbow trout intestinal bacterium (T1 &D22)	AY374104 AY374116	97 99	1487
8	24	Uncultured *Streptococcus sp*	AY307987	98	842
5	15	Uncultured bacterium clone HuCB85	AJ409007	97	1513

BV samples, having 97 and 99% identity respectively. The gene bank accession number of the two clones of Rainbow trout intestinal bacterium range from AY374104 to AY374116, having DNA sequence length base pair of 1487.

Discussion

The present study using PCR-based methods revealed that over 90% of the samples were colonized by more than one organism as shown in the DGGE bands. The findings support previous studies on the polymicrobial nature of BV patients [16]. Although *Mycoplasma hominis* is regarded as a causative agent in BV [17], the present study suggests that it may be more prevalent in Nigerian women than Caucasians. The eubacterial primers used here had previously been successful in detecting more common BV isolates in Caucasian women, namely *G. vaginalis, Prevotella* sp., *Mobiluncus*, *Atopobium* sp., as well as *E. coli* [10,18], none of which were detected in this population sample. A recent paper has suggested using a range of primers, including ones with up to 100 bases. While a range of primers and specific gene probes may have detected isolates present in low counts, we are confident that none of the Gram negative bacteria or *Atopobium* commonly associated with BV were present here as dominant organisms.

Cultivation of mycoplasma is rarely performed in Nigerian clinics or teaching hospitals, while gene-based diagnostics are not used at all. If the present sampling is indicative of a larger prevalence of mycoplasma-associated BV, it raises the question about detection and effective treatment of this condition. Already, many women self-diagnose vaginal symptoms as yeast infections and then self-medicate with over-the-counter antifungals, when in fact they have BV [19]. This misdiagnosis and subsequent mistreatment may result in adverse consequences [20].

More importantly, as BV raises the risk of sexually transmitted infection, including HIV [21], failure to detect it could have serious consequences to Nigerian women already at high risk for acquiring HIV. The use of gene-based methods often uncovers microbial types that are either not classified into a known genera, or are regarded as being unusual for the habitat sampled. This was the case here, with the detection of an organism identified by the gene bank as a Rainbow trout intestinal bacterium. This organism is normally associated with marine ecosystems, which suggests that it could have colonized the hosts through food consumption and ascension from the rectum to the vagina, or through transfer directly into the vagina after swimming in a river or lake. At least a portion of the organisms found in the microbiota of fish appear capable of persisting in the human intestinal tract [22]. On the other hand, it is possible that the primers used or the sequencing profiles have either mistaken this organism for Gram negative bacteria in previous studies, or we have mis-identified it here and the clones are in fact more commonly isolated enterobacteriaciae. The genera belongs to the Gamma sub-class of proteobacteria, and the clone TI is very close to *Enterobacteriaceae* in the phylogenetic group, while D22 is closely related to *Proteus sp*. [23]. A number of intestinal fish microbes, such as *Aeromonas sp.*, do express virulence factors and can potentially infect humans [24], however, none have previously been associated with BV. It remains to be determined the extent to which the trout organisms isolated here do in fact colonize the vagina, displace lactobacilli and induce a symptomatic BV condition, or whether they are simply commensal

bystanders.

In conclusion, this study has revealed the polymicrobial nature and high prevalence of mycoplasma-based BV in Nigerian women. The application of gene-based methodologies to countries like Nigeria is dependent upon money being available and personnel being trained to perform and interpret the resultant data. For this population, the consequences of misdiagnosis of vaginal infections may have major health implications.

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