

Anaerobe 2010

The 10th Biennial Congress of the
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USE OF MOLECULAR PROFILING TO DESCRIBE THE SPECTRUM AND DYNAMICS OF VAGINAL MICROBIOTA: AN UPDATE

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Bacterial vaginosis (BV) affects millions of women annually, may produce malodorous vaginal discharge, and is associated with several adverse health outcomes including pre-term birth, HIV acquisition, and pelvic inflammatory disease. The cause of BV is not known, though changes in vaginal bacterial communities are evident with a shift from a *Lactobacillus* dominated bacterial biota to one dominated by *Gardnerella vaginalis* and several anaerobes. The recent use of molecular, cultivation independent methods to study the vaginal microbiota has revealed highly complex vaginal bacterial communities in women with BV, with a significant increase in species richness and diversity compared to the vaginal bacterial communities in women without BV. Several of the vaginal bacteria detected by PCR in women with BV are either difficult to cultivate in the laboratory or difficult to identify using conventional phenotypic algorithms. Studies using quantitative PCR to measure concentrations of key vaginal bacteria provide evidence that the vaginal microbiota can be highly dynamic, with rapid shifts in bacterial concentrations in response to endogenous host factors (menses) and external factors such as sexual activity. Intra-vaginal metronidazole therapy for BV results in rapid declines of BV-associated bacteria in the vagina, whereas relapse of BV is associated with re-emergence of these bacteria, suggesting that antibiotic resistance is not the most important factor mediating treatment failure. BV is a polymicrobial infection that responds to antibiotics with anti-anaerobic bacteria activity but has a very high rate of relapse. The pathogenesis of BV remains poorly understood, but molecular tools are providing exciting new insights into this common condition.

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EASIER TO GROW THAN TO IDENTIFY: CHARACTERIZATION OF NOVEL BACTERIA FROM THE FEMALE GENITAL TRACT

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While broad range culture-independent methods have identified a number of novel bacteria which have not previously been detected by culture, these methods do not routinely detect some of the common organisms which are known to be pathogens including Group B *Streptococcus*. Advantages of culture dependent methods include the capacity to identify pathogens using selective media at low cost, the availability of the bacteria for antibiotic susceptibility testing and phenotypic characterization and full genomic sequencing which could lead to identification of factors associated with virulence. However, some vaginal bacteria including *Sneathia* and *Leptotrichia* are rarely recovered by culture. Other bacteria can be cultivated on agar media but cannot be identified using currently available taxonomic tests. These unidentified bacteria are typically clustered into phenotypic groups such as “anaerobic gram negative rods” or anaerobic gram positive cocci”. Thus, some organisms which can be grown on agar media have been considered to be noncultivable when detected by culture independent methods because there are no available methods which can be used to identify them after their isolation on culture media. One example of such an organism is the anaerobic gram positive rod in the Clostridiales family, BVAB3, which has been provisionally named *Mageebacillus indolicus*. In order to gain a more comprehensive understanding of the genital microflora, it is likely that a combination of culture-dependent and sequencing techniques will be needed. Recently, a combination of biochemical tests, 16S rDNA gene sequencing, and restriction fragment length polymorphism (RFLP) has been used for identification of bacteria grown on culture media from reproductive tract specimens. Based on a combination of culture and sequencing methods, a broad diversity of bacteria has been identified. For example, in a study of 85 pregnant women, 932 isolates representing 152 different species were recovered, including 16 novel species. The mean number of isolates per woman were significantly higher in women with bacterial vaginosis (18.6) compared to intermediate flora (12.5) and normal flora (8.1). A direct comparison of culture-based methods to results from culture independent methods may help define the clinical relevance and comparative costs of these methods for describing the role of these bacteria in disease and health.

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INTERPRETING THE EPIDEMIOLOGY AND NATURAL HISTORY OF BACTERIAL VAGINOSIS: ARE WE STILL CONFUSED?

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Bacterial vaginosis (BV) is a common cause of vaginitis and increases women's risk of pelvic inflammatory disease, adverse pregnancy outcomes, and risk of STD/HIV acquisition. The etiology of BV is unclear, though it is believed to involve loss of vaginal hydrogen peroxide-producing lactobacilli and acquisition of complex bacterial communities that include many fastidious BV-associated bacteria (BVAB) that have recently been detected using PCR methods. Treatment failure (persistence) is common, and may be facilitated by unprotected sex. Potential contributions to BV and BV persistence include (1) sexual partners as a reservoir for BVAB; (2) specific sexual practices, including male partners' condom use; and (3) the composition of the vaginal microbiota involved in BV. Specific BVAB in the Clostridiales Order may predict BV persistence when detected pre-treatment, and have been detected in men whose female partners have BV. BVAB may be associated with unprotected sexual behavior and failure of BV to resolve in women, supporting the hypothesis that BVAB colonization of male genitalia may serve as a reservoir for re-infection of female partners. Moreover, specific sexual practices may favor vaginal colonization with certain BVAB that have been associated with persistence. This presentation will review the epidemiologic and microbiologic data to support a role for acquisition of BVAB and how this process might differ among subsets of women.

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VALIDATION OF ALGORITHM FOR IDENTIFICATION OF *PREVOTELLA* SPECIES FROM THE FEMALE GENITAL TRACT USING 16S rDNA PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

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Objective: Organisms in the genus *Prevotella* are commonly isolated from the genital tract of women with bacterial vaginosis. Species identification using phenotypic characteristics and/or enzymatic activity is imperfect and time consuming. Classification based wholly on sequencing, while faster and more specific, is expensive. Our objective was to develop and validate a rapid and reproducible identification algorithm for *Prevotella* species using 16S rDNA PCR-RFLP.

Methods: DNA was extracted from 19 reference strains of *Prevotella* and 415 bile sensitive, obligately anaerobic gram negative rods isolated from the genital tract of 131 pregnant women. Following broad range 16S rRNA gene PCR, amplified 16S rRNA genes were digested singly using *Hae* III and fragments were resolved on 3% high resolution gels. Patterns generated from the clinical strains were compared to reference strain patterns and assessed independently by two evaluators to determine identity. PCR products from five of each pattern generated were sent for direct sequencing to validate and confirm pattern identification.

Results: *Hae* III digestion of 19 reference strains resulted in 17 distinct patterns. Sequencing of five clinical isolates for each pattern observed confirmed species identification and matched the corresponding reference strain pattern. There was 98.3% agreement between the two evaluators. *P. bivia* (24.6%) and *P. timonensis* (24.1%) were the species most commonly isolated followed by *P. buccalis*/*oulorum* group (16.6%), *P. amnii* (16.1%), *P. disiens* (5.1%), *P. melaninogenica*/*histicola* group (2.2%), *P. corporis* (1.2%), and *P. bergensis* (0.7%). Three novel *Prevotella* with sequences and patterns not matching any validly published species were also observed and are referred to as unknown *Prevotella* group 1 (4.6%), group 4 (0.96%) and group 9 (2.2%).

Conclusion: 16S PCR-RFLP is a simple, rapid, and reproducible method for determining species identification of most organisms belonging to the genus *Prevotella* and can be used to distinguish novel species. A limitation of this study is the indistinguishable pattern observed among *P. buccalis* and *oulorum* and *P. melaninogenica* and *histicola* and additional testing for characterization will be needed.

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IDENTIFICATION OF ANAEROBIC GRAM POSITIVE RODS FROM THE FEMALE GENITAL TRACT USING 16S RDNA PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPS)

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Background: Classification of anaerobic gram positive rods (AGPR) using conventional phenotypic characteristics or enzymatic activities is unreliable as many species are indistinguishable biochemically. Phylogenetic analysis of 16S rRNA genes of organisms belonging to this group allows differentiation, but can be costly. Our objective was to test the feasibility of identifying AGPR recovered from the female genital tract using 16S rDNA PCR-RFLP.

Methods: 189 AGPR recovered from the female genital tract were subjected to DNA extraction followed by broad range 16S rRNA gene PCR using universal primers. Restriction endonuclease digestions of amplified genes using *HaeIII* were subjected to electrophoresis on 3% high resolution gels. Direct sequencing of the PCR product from up to 5 representative isolates with the same profile confirmed the identification. Validated profiles were then used as reference for identifying the remaining clinical isolates.

Results: 15 unique RFLP patterns were observed following digestion with *HaeIII* and corresponded to 16 different species from 13 genera. With the exception of the *Propionibacterium acnes*/unclassified organism group we were able to determine the difference between organisms using a single restriction enzyme. Of 190 isolates, 52% were *Atopobium* species, followed by *Mobiluncus* species (18%), *Propionimicrobium lymphophilum* (10%), *Propionibacterium acnes*/Unclassified organism (7%), *Actinomyces hongkongensis* (5%), *Collinsella aerofaciens* (2.1%), *Actionbaculum* species (2%), BVAB3 (1%), *Solobacterium morrei* (1%), BVAB2 (0.5%), *Clostridium scindens* (0.5%), and *Leptotrichia amnionii* (0.5%).

Conclusion: Using a single restriction enzyme to classify AGPR is more cost efficient than sequencing only and more accurate than utilizing phenotypic characteristics for identifying isolates from the female genital tract. Profiles of multiple species in each genus will need to be observed to confirm that a single restriction enzyme can be used for the AGPR group, or if a second enzyme is needed.

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IDENTIFICATION OF ANAEROBIC GRAM POSITIVE COCCI IN THE FEMALE GENITAL TRACT USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS OF THE 16S RDNA

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Objective: Anaerobic gram positive cocci (AGPC) are common bacteria found in the vagina and endometrium; however, their identification to the species level is difficult. This study assessed the use of RFLP analysis for identifying AGPC.

Methods: 458 clinical isolates obtained from the female genital tract were identified as AGPC by aero-tolerance and Gram stain. DNA was extracted from pure colonies of each isolate and 14 ATCC type strains of AGPC using Prepman™ Ultra, and the 16S rRNA region was amplified by PCR. The restriction enzyme *HinfI* was used to digest the PCR product and distinct banding patterns were observed on a 3% high-resolution agarose gel. Restriction digest gels of the unknown isolates were compared to the patterns of the AGPC type strains. To confirm species identification by RFLP, sequences of 500 base pairs of the 16S rDNA from at least 5 clinical isolates for each species were compared with GenBank using the basic local alignment search tool (BLAST).

Results: Based on the *HinfI* restriction enzyme, all 458 isolates were identifiable to the genus level and 71% to the species level. Species-specific banding patterns were observed for 9 of the 14 AGPC type strains, and the remaining 5 were grouped into the genus *Anaerococcus vaginalis/hydrogenalis*, *Peptoniphilus indolicus/asaccharolyticus*, and *P. harei*/unknown species. Of the 458 clinical isolates, 325 AGPC were identified by *HinfI* to 8 different species; *Finegoldia magna* (26%), *A. tetradius* (18%), *P. lacrimalis* (17%), *Peptostreptococcus anaerobius* (4%), *A. lactolyticus* (2%), *P. ivorii* (2%), *Peptococcus niger* (1%), and *A. prevotii* (1%). Fourteen (3.1%) AGPC were identified as *A. vaginalis/hydrogenalis* and 119 (26%) were identified as *P. harei*/unknown species. Sequence analysis confirmed the species identification of at least 5 clinical isolates for each RFLP pattern observed.

Conclusion: RFLP analysis using *HinfI* has shown to be a feasible method for identification of all genera of AGPC and 71% of the species. In a laboratory setting, this identification system is a relatively simple, rapid tool that may also aid in the discovery and identification of novel species, and the misidentification of other bacteria morphologies as AGPC. Further analysis is needed to expand this identification scheme to include AGPC species other than the 14 type strains tested.

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DESCRIPTION OF A NOVEL *PEPTONIPHILUS* SPECIES FROM THE FEMALE GENITAL TRACT

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Objective: To describe a novel species of anaerobic gram positive cocci (AGPC) recovered from vaginal and endometrial specimens.

Methods: 119 vaginal and endometrial isolates were recovered from Brucella agar supplemented with 5% sheep blood and identified as AGPC by Gram stain and aero-tolerance. DNA was extracted using Prepman™ Ultra from pure colony isolates and 14 ATCC type strains of AGPC and the 16S rRNA was amplified by PCR. PCR product from all 119 clinical isolates and the 14 AGPC type strains were digested with the restriction enzyme *HinfI* using restriction fragment length polymorphism (RFLP) analysis. Distinct banding patterns were observed on a 3% high-resolution agarose gel and compared with the AGPC type strain patterns. 18 representative clinical isolates with the same RFLP pattern were sequenced using 500 base pairs of the 16S rDNA and 5 were characterized biochemically.

Results: Based on the *HinfI* restriction pattern, all 119 clinical isolates had the same banding pattern as *Peptoniphilus harei*. However, sequencing data showed only 96% similarity to *P. harei* and other species of *Peptoniphilus*. The closest match in GenBank was to *Peptoniphilus* species (Accession AY244778 and AY244779) at 97-98%. All of the 5 isolates characterized biochemically were positive for the production of indole, negative for urease, alkaline phosphatase, α -galactosidase, β -galactosidase, α -glucosidase, and catalase, and had a major terminal volatile fatty acid product of butyrate. Biochemical results were variable for the fermentation of glucose and the activity of arginine arylamidase and pyrrolidonyl arylamidase. This organism was not distinguishable biochemically from other AGPC phenotypes.

Conclusion: Although this AGPC has been isolated previously and sequences have been deposited in GenBank; this organism has not been classified to the species level. This novel species is commonly found in the female genital tract; however, its significance remains unknown. Bio-chemical composition and sequence analysis indicates this organism may be an indole positive *Peptoniphilus* species; however, further investigation is needed to fully identify this novel species.

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CHARACTERIZATION OF VAGINAL LACTOBACILLI IN WOMAN AFTER KIDNEY TRANSPLANTATION

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In kidney transplant and hemodialyzed women, immunity suppression related to primary disease and immunosuppressive therapy results on changes of vaginal physiologic microflora. The aim of this study was to evaluate the prevalence of vaginal lactobacilli types in kidney transplant and hemodialyzed patients compared with healthy women.

Vaginal swabs taken from 20 women of the study group and 20 women of control group were investigated. Each women was tested for bacterial vaginosis according to Amsel and Nugent criteria. Swabs were cultured on MRS agar and Columbia blood agar (bioMerieux, Marcy L'Etoile, France) in high CO₂ and anaerobic conditions at 37° C for at least 48 hours. Colonies were evaluated by morphology, Gram staining, hemolytic and catalase activities, H₂O₂ production abilities (according to Eschenbach, 2003) and biochemical properties (API 50 CHL, bioMeriux, Marcy L'Etoile, France). For further PCR identification 41 strains of *Lactobacillus* spp. were evaluated, multiplex PCR was performed by Song et al, 2001.

No bacterial vaginosis (1 or 2 in Nugent scale) was diagnosed among women of study and control groups. No significant differences in H₂O₂ production abilities between lactobacilli isolated in study group and healthy women was demonstrated.

Only 20% of concordance between phenotypic (API 50CHL) and genotypic (mPCR) identification of lactobacilli was demonstrated: the highest among *L. crispatus* (about 50%).

Out of 40 lactobacilli evaluated in mPCR only 35 were identified (15 from study group and 20 from control group). Among identified strains occurrence of *L. gasseri* and *L. delbrueckii* was the same in study and control groups. *L. crispatus* strains were isolated 2 time more often in control group than in the study group. Five strains isolated from vagina of study group women were not identified by mPCR.

MIC determination for erythromycin, clindamycin, tetracycline, moxifloxacin and metronidazole did not demonstrate any differences between sensitivity profile and resistant strains were not isolated.

We concluded that differences in lactobacilli amount and types in study and control groups were not evident. However it is possible that unidentified strains represented *L. iners* which can be important for immunocompromised patients. Further studies will be helpful for explanation of these findings.

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IDENTIFICATION AND HYDROGEN PEROXIDE PRODUCTION OF VAGINAL LACTOBACILLUS FROM WOMEN WITH OR WITHOUT BACTERIAL VAGINOSIS

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Lactobacillus spp., principally hydrogen peroxide (H₂O₂) producing strains, may have a protective effect against vaginal colonization by pathogenic microorganisms such as those that cause bacterial vaginosis. In the present study, we report and compare the identification and H₂O₂ production of *Lactobacillus* isolated from vagina of women with or without bacterial vaginosis. Bacteria were isolated onto the Man, Rogosa and Sharpe agar medium (MRS), and identified at species level by PCR amplification of 16S-23S rRNA intergenic spacers followed by restriction digestion analyses of PCR products. H₂O₂ production was determined by a qualitative method using the TMB-plus medium (*Brucella* agar base, 3,3',5,5'-tetramethylbenzidine, horseradish peroxidase, starch, vitamin K, hemin, magnesium sulfate, manganese sulfate, and horse serum). The most commonly isolated species were *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. johnsonii*. Amounts of H₂O₂ produced by *Lactobacillus* varied widely as evaluated by color intensity of the indicator. Among 68 strains studied, 33 produced H₂O₂ and 7 of them showed the highest production. These *Lactobacillus* producing high levels of H₂O₂ (essentially *L. jensenii* and *L. johnsonii*) were more frequently present in the vagina of healthy women. Concluding, we postulate that H₂O₂-producing *Lactobacilli* are able to reduce the incidence of vaginal infections such as bacterial vaginosis.

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MICROBE-SPECIFIC MODULATION OF INFLAMMATORY RESPONSE IN EXTREMELY LOW GESTATIONAL AGE NEWBORNS

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The fetal response to intrauterine inflammatory stimuli appears to contribute to the onset of preterm labour, and fetal injury in extremely low gestational age newborns. This study demonstrates that the inflammatory response to bacterial colonization of the very preterm placenta is microorganism specific. We analysed 27 protein biomarkers in dry blood spots from 527 newborns born by cesarean section in the 23rd - 27th gestation weeks. Bacteria were detected in placentas and characterized by culture techniques. Odds ratios of having protein concentrations in the top quartile were calculated for individual and groups of microorganisms. Mixed bacterial vaginosis (BV) organisms, detected in 13% of the placentas, were associated with proinflammatory patterns similar to those of infectious facultative anaerobes. *Prevotella*, *Gardnerella*, anaerobic streptococci, peptostreptococci, and genital mycoplasmas each appeared to provoke a different pattern of elevated concentrations of inflammation-related proteins in the newborn's blood. *Lactobacillus* detected in 6% of the placentas was associated with low odds of inflammatory responses and appeared to suppress inflammatory responses when mixed with other microorganisms. These results support concepts of the beneficial role of *Lactobacillus* and the targeting of early pregnancy placental colonization by specific drugs or probiotics as a strategy for prevention of inflammation-provoked sequelae in the premature new-borns.