UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

ASPECTOS CLÍNICOS, MICROBIOLÓGICOS E DA REGULAÇÃO DA IMUNIDADE INATA NA VAGINOSE BACTERIANA

Camila Marconi

Tese apresentada ao Programa dePós-Graduação em Patologia da Faculdade deMedicina deBotucatu, Universidade EstadualPaulista – UNESP para obtenção do título deDoutor em Patologia.

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Orientadora: Profa. Dra. Márcia Guimarães da Silva

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Sumário

1. Revisão da literatura

A vaginose bacteriana (VB) é considerada a alteração de microbiota vaginal mais comum entre as mulheres em idade reprodutiva.[1,2,3] É caracterizada pela substituição da microbiota vaginal normal, constituída predominantemente por *Lactobacillus* spp., pelo crescimento de outras espécies bacterianas, em sua maioria anaeróbias.[4] Portanto, a etiologia da VB é considerada como polimicrobiana, já que seu desencadeamento não está relacionado a um único microrganismo, mas a um conjunto heterogêneo de espécies bacterianas.

Embora a VB seja considerada a mais frequente alteração de microbiota vaginal, sua frequência varia de 5,8% a 24,4%, dependendo da população estudada.[5] Nos Estados Unidos, a VB está presente em 29,2% das mulheres da população geral, sendo que sua frequência na população americana varia conforme a etnia, de 23,2% em brancas não hispânicas a 52,4% em afro-descendentes.[1] Estudos brasileiros realizados com objetivo de avaliar infecções do trato genital inferior (TGI) em gestantes demonstraram frequências de cerca de 12% de VB em pacientes assintomáticas[6,7] a 29% em gestantes atendidas em ambulatório de primigestas.[1]

A sintomatologia da VB é variável e pode incluir corrimento vaginal, mau odor genital durante o período menstrual ou após relação sexual, além de prurido. No entanto, um dos principais problemas relacionados ao diagnóstico desta alteração é a grande porcentagem de pacientes assintomáticas.[5,8] Esta observação se torna de fundamental importância já que muitos estudos demonstraram que a VB é fator de risco para inúmeras complicações ginecológicas[9-13] e obstétricas.[14-17]

Atualmente, o Ministério da Saúde[18] recomenda que o diagnóstico de VB seja realizado através da presença de três ou mais dos critérios estabelecidos por Amsel et al.[19] que compreendem: corrimento vaginal branco homogêneo, pH vaginal maior que 4,5, whiff *test* positivo e presença de *clue cells* no exame microscópico. Outro método recomendado e amplamente utilizado para o diagnóstico da VB é análise microscópica do esfregaço vaginal corado pelo método de Gram, conforme o sistema de escores preconizado por Nugent et

al.[20]Mais recentemente, outro método foi descrito por Donders et al.[21] para avaliação da microbiota vaginal através da análise de esfregaços a fresco por microscopia de contraste de fase.

Inúmeras complicações obstétricas e ginecológicas são associadas à VB. Em relação ao desenvolvimento de VB durante a gestação, é conhecido que esta alteração de microbiota vaginal é fator de risco para várias complicações como aborto espontâneo,[14] corioamnionite,[15] trabalho de parto pré-termo,[16] parto pré-termo e, como conseqüência, baixo peso ao nascimento.[17] Dentre as intercorrências ginecológicas destacam-se a doença inflamatória pélvica[9] e as infecções pós-cirúrgicas.[10]

Estudos recentes também demonstraram associação da VB com aquisição de Doenças Sexualmente Transmissíveis (DSTs), dentre elas cervicites por *Chlamydia trachomatis* (CT) e *Neisseria gonorrhoeae*,[11] tricomoníase[12]e infecção pelo vírus da imunodeficiência humana (HIV).[13] Muitos fatores podem estar envolvidos no mecanismo pelo qual a presença de VB facilita a aquisição dessas DSTs. O desbalanço na imunidade local causado pela alteração da microbiota pode causar danos teciduais e consequente aumento da suscetibilidade do tecido aos microrganismos associados às DSTs.[22] Além disso, produtos bacterianos são capazes de diminuir a viscosidade da secreção cervical, levando a prejuízo da primeira linha de defesa local contra patógenos.[23]

Estudo recente descreve que não apenas a VB aumenta o risco de aquisição de *C. trachomatis*, mas a presença dessa cervicite também pode levar ao desenvolvimento de VB,[24] indicando, portanto, que a CT também pode estar envolvida em alterações vaginais levando a um desequilíbrio desse ambiente. Em estudos recentes, demonstramos que a frequência de infecção clamidiana é alta em população de alto risco da região de Botucatu e está associada à microbiota vaginal alterada.[25,26]

Com relação à instalação da VB, já foram descritas variáveis que podem predispor ao seu estabelecimento como hábitos sexuais, duchas vaginais e estresse crônico.[27,28] No entanto,

ainda não se sabe qual o fator desencadeante da transição da microbiota vaginal normal para a VB, ou seja, ainda não foi estabelecido se o desaparecimento das espécies de lactobacilos é um evento precedente à instalação da VB ou se esta é conseqüência do crescimento exagerado de outras espécies bacterianas.

Assim como o fator desencadeante da VB, outros aspectos relacionados à sua fisiopatologia ainda permanecem desconhecidos. No entanto, sabe-se que a colonização do TGI por espécies de lactobacilos é considerada de fundamental importância para a manutenção da saúde vaginal e maior resistência a infecções bacterianas e virais, inclusive ao HIV.[12,13,29,30,31]

O epitélio vaginal, sob ação estrogênica, acumula glicogênio que posteriormente será hidrolisado em glicose e metabolizado pelos lactobacilos presentes na microbiota vaginal[26]. O ácido lático derivado dessa via metabólica leva à redução do pH vaginal em níveis considerados normais, ou seja, entre 3,8 a 4,5.[32] O pH ácido, em conjunto com outros fatores como a liberação de peróxido de hidrogênio e bacteriocinas pelas espécies de lactobacilos, desempenham papel fundamental para a inibição do crescimento de outras espécies bacterianas.[33] Vários outros gêneros como *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Gardnerella*, *Bacteroides*, *Enterococcus* e *Mycoplasma*podem estar presentes na microbiota vaginal normal, desde que em menor quantidade que as espécies de lactobacilos.[34,35]

Muitas espécies bacterianas podem ser isoladas do conteúdo vaginal de mulheres com VB, sendo observada grande diversidade entre as espécies isoladas nos diferentes casos.[36] Durante as últimas décadas,vários estudos foram realizados na tentativa de definir o padrão de espécies bacterianas associadas à VB utilizando cultura do conteúdo vaginal.[36,37] Embora alguns microrganismos tenham sido associados à VB, como *Gardnerella vaginalis*,[38] *Mobiluncus curtisii,*[39] *Mycoplasma hominis,*[40]essas espécies também podem ser encontradas colonizando o TGI de mulheres com microbiota normal,[37,41] portanto o simples isolamento dessas espécies

não é útil para o diagnóstico de VB. Além disso, deve-se ressaltar que estudos que utilizaram a cultura como método para avaliação microbiológica da VB podem ter apresentado resultados incompletos quanto a real composição da microbiota vaginal, já que apenas pequena porcentagem de espécies bacterianas pode ser cultivada em laboratório.[42,43]

Considerando que muitos aspectos relacionados ao desenvolvimento da VB ainda são desconhecidos, é possível que grandes avanços sejam possíveis com base nos resultados de estudos recentes, empregando técnicas moleculares para detecção e quantificação dos microrganismos presentes na microbiota vaginal.[42,44,45] Esses estudos já demonstraram que existe maior diversidade dentre as espécies bacterianas presentes em casos de VB quando comparados à microbiota vaginal normal.[45] Além disso, verificou-se que existe correlação entre contagem bacteriana total presente no conteúdo vaginal, em Unidades Formadoras de Colônia (UFC), e o *score* dos esfregaços vaginais, segundo a classificação de Nugent.[46]

Os métodos moleculares utilizados para caracterização dos microrganismos presentes na microbiota vaginal permitiram a identificação de inúmeras espécies bacterianas até então nunca detectadas em culturas do conteúdo vaginal.[44,45] Somado a isso, foram encontradas novas espécies de microrganismos associadas à VB, como *Atopobium vaginae*, *Leptotrichia* sp., *Megasphaera* sp., entre outros. Apesar de relevantes as informações quanto à associação dessas espécies aos casos de VB, deve-se ressaltar que esses microrganismos também são detectados no conteúdo vaginal de mulheres apresentando padrão normal da microbiota vaginal.[44,45,46]

A realização de estudos empregando ferramentas de biologia molecular para identificação das espécies presentes nos casos de VB, permitiu grande avanço para determinação exata dos microrganismos presentes nesse ambiente. Recentemente foi demonstrado que o *core* associado à VB é altamente variável entre as mulheres e que a composição bacteriana no mesmo indivíduo é bastante dinâmica.[47] Em estudo realizado através de clonagem e sequenciamento para identificação das espécies bacterianas em

mulheres sem qualquer infecção do TGI, Zhou et al.[42] demonstraram que, nem sempre, os *Lactobacillus* sp são predominantes nessa população, já que foram observadas microbiotas vaginais com predominância de espécies associadas à VB como *Atopobium vaginae*, *Leptotrichia* sp. e *Megasphaera* sp. Extremamente relevante é a informação de que essas espécies são produtoras de ácido lático, componente fundamental do microambiente vaginal, por reduzir o pH e, conseqüentemente, inibir o crescimento de microrganismos potencialmente patogênicos.[42,48] Dessa forma, Zhou et al.[42] afirmam que a manutenção do pH ácido apresenta maior importância para a conservação da microbiota vaginal normal do que a diversidade das espécies bacterianas em sua composição. Com base nesses achados, Witkin et al. [49] sugerem que essa seja a razão pela qual mulheres, que apesar da ausência de lactobacilos nos esfregaços vaginais, se apresentam assintomáticas.

A espécie *Atopobium vaginae* foi descrita somente em 1999.[48] Esse microrganismo Gram-positivo tem morfologia variável, de cocos alongados até bacilos, e podem se apresentar isolados, em pares ou em pequenas cadeias. Crescem em placas de ágar-sangue incubadas em anaerobiose a 37^{o}C como pequenas colônias, porém seu isolamento a partir de materiais clínicos é difícil. Embora já tenha sido verificada sua presença na microbiota de mulheres normais,[42] muitos estudos têm demonstrado que o *A. vaginae* é mais frequentemente encontrado na microbiota vaginal de pacientes com VB e têm sugerido importante papel dessa espécie na patogênese dessa intercorrência.[44,50] A detecção do *A. vaginae* no conteúdo vaginal apresenta alto valor preditivo para o diagnóstico de VB,[44,45,51] principalmente quando há co-detecção de *Gardnerella vaginalis.*[44] Outro importante aspecto relacionado ao *A. vaginae* é a grande porcentagem de pacientes que apresentam recorrência da VB quando na sua presença,[51] o que pode ser explicado pelas altas taxas de resistência apresentadas por essa espécie ao metronidazol, tratamento mais utilizado para a VB.[52]

As recentes análises das sequências do gene RNAr 16S dos microrganismos presentes no conteúdo vaginal também permitiram a identificação de novas espécies presentes na

microbiota vaginal de mulheres com VB, como *Megasphaera* sp. e *Leptotrichia* sp.[45] Essas espécies, assim como o *A.vaginae*, também são produtoras de ácido lático. Além da associação de *Megasphaera* sp. e *Leptotrichia* sp. com a VB, Fredricks et al.[53] demonstraram que a presença desses microrganismos, somada ao *Atopobiumvaginae*, apresenta maiores valores de sensibilidade para o diagnóstico de VB quando comparada aos critérios de Amsel et al.[19] e Nugent et al.[20] que são amplamente utilizados. Entretanto, Witkin et al.[49] propõem que essas espécies, da mesma forma que os lactobacilos, são capazes de manter o pH vaginal ácido, protegendo contra a proliferação de microrganismos patogênicos. Portanto, mulheres que apresentam microbiota com ausência de lactobacilos, mas com pH vaginal ácido mantido por outras espécies produtoras de ácido lático, não deveriam ser incluídas no grupo de pacientes com VB clássica.

Considerando a realidade atual da maioria dos laboratórios de diagnóstico, a utilização de métodos moleculares, como a técnica de PCR quantitativo, não pode ser considerada opção para o diagnóstico da VB, já que não possui fácil execução e apresenta altos custos quando comparada aos métodos preconizados por Amsel et al.[19] e Nugent et al.[20] No entanto, tendo em vista a grande importância da avaliação quantitativa das espécies bacterianas associadas à VB para a melhor compreensão de sua fisiopatologia e possíveis prejuízos à saúde da mulher, a técnica de PCR quantitativa constitui a melhor opção metodológica para a realização de estudos com esse objetivo.[54]

Alguns autores sugerem que o desenvolvimento da VB esteja diretamente relacionado à imunidade inata local.[55,56] Tem sido proposto que a presença de alguns produtos bacterianos presentes na microbiota vaginal alterada inativem enzimaticamente os receptores *toll-like* (TLRs) presentes na superfície das células epiteliais, gerando uma resposta inflamatória ineficaz.[56] Os TLRs, quando estimulados por produtos microbianos, levam a ativação de NF-kB, um fator de transcrição que está envolvido na indução de síntese e liberação de citocinas inflamatórias.[57] Dentre as dez isoformas de TLRs já identificados nos tecidos humanos,[57] os

tipos 2 e 4 são de fundamental importância para a resposta à infecção bacteriana, pois reconhecem, respectivamente, as lipoproteínas presentes na parede de gram-positivos e micoplasmas[58,59] e o lipopolissacarídeo de bactérias gram-negativas.[60] Corroborando essa hipótese, já foi demonstrado que o polimorfismo no gene que codifica o TLR tipo 4, que resulta em menor atividade desse receptor, foi associado com o desenvolvimento de VB.[61]

Inúmeros trabalhos têm relacionado níveis aumentados de citocinas inflamatórias com resultados adversos da gestação[62,63] e aumento da infectividade ao HIV.[64,65] Pode-se afirmar, portanto, que o aumento exacerbado da produção de citocinas em resposta à VB predispõe a paciente às complicações mais severas dessa alteração de microbiota vaginal.

A Interleucina (IL)-1beta é a principal citocina envolvida na resposta imune inata das infecções do TGI e suas complicações relacionadas.[62,66] Esta é a primeira citocina liberada em resposta a componentes bacterianos e induz a secreção de outras citocinas inflamatórias por células epiteliais do TGI.[67,68] Vários estudos já demonstraram que níveis aumentados dessa citocina são encontrados no conteúdo vaginal de mulheres com VB.[69,70,71]

Já é bem estabelecido que a IL-1β induz secreção de IL-6, IL-8 e TFN-α por células do epitélio vaginal.[67] No entanto, os resultados encontrados em estudos que avaliaram essas citocinas no conteúdo vaginal de mulheres com VB são contraditórios. Com relação ao TNF-α, não são verificados aumentos significativos de seus níveis em pacientes com microbiota vaginal alterada[70] porém alguns autores demonstraram haver maior produção dessa citocina, por células epiteliais, quando estimuladas com sobrenadante de conteúdo vaginal na presença de VB,[65] sugerindo possível mecanismo pelo qual há aumento da transmissão de microrganismos associados a STIs na presença da VB.[22]

Os níveis de IL-8 também já foram demonstrados estar elevados na presença de VB,[70] no entanto, outros autores demonstraram que, diferente do que ocorre com a IL-1β, não há correlação entre VB, aumento de IL-8[69,71] e número de neutrófilos no conteúdo vaginal.[69]

Níveis inalterados da IL-6 são encontrados na VB em relação a mulheres normais,[70,71] entretanto, já foi demonstrado que células epiteliais *in vitro* secretam maiores níveis dessa citocina em resposta a microrganismos associados à VB como a *G. vaginalis* e o *A. vaginae.*[72]

Recentementefoi demonstrado que níveis aumentados de IL-1β na VB se correlacionam com o aumento de atividade das enzimas bacterianas, as sialidases, que são produzidas por várias bactérias anaeróbias associadas à VB.[73] As sialidases são enzimas que clivam o ácido siálico de glicoproteínas, dentre elas glicoproteínas que compõe a imunoglobulina A secretada no local e, dessa forma, está associada à evasão da imunidade vaginal.[74]

Atualmente, vários autores têm apontado para uma grande heterogeneidade na resposta imunológica do hospedeiro à VB.[49,70,75] Sendo assim, mais informações quanto à constituição da microbiota vaginal e a sua relação com a modulação da resposta inflamatória são necessárias para definir os mecanismos da fisiopatologia da VB e das complicações associadas a ela. Com base nesse conhecimento, seria possível esclarecer quais os parâmetros que devem ser adotados para definir essa alteração de microbiota vaginal e permitir seu tratamento mais efetivo.

Com base na grande diversidade microbiológica presente nos casos de VB e nos recentes achados quanto a sua associação com as espécies consideradas produtoras ácido lático, *A. vaginae., Megasphaera* sp. e *Leptotrichia* sp., tem sido sugerido que a microbiota vaginal apresentando predominância dessas espécies não deva ser classificada como anormal.[49] Considerando que as complicações advindas da VB estão relacionadas com a resposta imune local, bem como a maior atividade enzimática bacteriana, a avaliação desses parâmetros dentre os casos de VB de acordo com a predominância dessas espécies pode contribuir para o entendimento da fisiopatologia da VB. Além disso, tendo em vista o importante papel da VB como fator facilitador da aquisição de DSTs, a avaliação da variação

resposta imune inata dentre os casos de VB é importante para definir qual o papel da microbiota vaginal na proteção contra patógenos.

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3. Objetivos

Considerando a alta prevalência de vaginose bacteriana e de infecção clamidiana em mulheres em idade reprodutiva, assim como o desconhecimento de muitos aspectos

relacionados à fisiopatologia dessas intercorrências ginecológicas, os objetivos desse trabalho foram:

3.1. Verificar se a participação *de Atopobium vaginae, Megasphaera* sp. e *Leptotrichia* sp. na vaginose bacteriana está associada a diferenças na produção de citocinas pró-inflamatórias, na atividade de sialidase e na quantidade bacteriana;

3.2. Determinar se a infecção clamidiana modula a produção de citocinas pró-inflamatórias na vaginose bacteriana.

4. Resumo

Introdução: A vaginose bacteriana (VB) é a alteração de microbiota vaginal mais frequente em mulheres em idade reprodutiva.Inúmeras complicações ginecológicas e obstétricas são

associadas à VB, como doença inflamatória pélvica, aumento do risco de aquisição de Doenças Sexualmente Transmissíveis (DSTs), corioamnionite clínica e histológica e baixo peso ao nascimento. A VB se caracteriza pela substituição dos lactobacilos da microbiota vaginal por outras espécies bacterianas, na sua maioria anaeróbias. Estudos recentes demonstraram que várias espécies até então raramente ou nunca isoladas em laboratório são associadas à VB como *Atopobium vaginae*, *Leptotrichia* sp. e *Megasphaera* sp. Essas espécies têm como característica comum a produção de ácido lático. Dessa forma, tem sido observado que mulheres assintomáticas podem apresentar ausência de lactobacilos na microbiota vaginal e predomínio de tais espécies. Portanto, alguns autores sugerem que elas possam contribuir para o equilíbrio do meio vaginal. Tendo em vista que os casos de VB apresentam grande heterogeneidade quanto à composição microbiológica, considera-se que a resposta imune também possa ser variável. A amplificação da resposta imune local na VB é um dos mecanismos que levam a maior suscetibilidade da mucosa vaginal à aquisição de DSTs, dentre as quais a infecção clamidiana que, é bastante frequente em nossa população. Embora estudos tenham demonstrado associação da infecção por *Chlamydia trachomatis* (CT) com a VB, poucos trabalhos avaliaram o perfil da resposta imune inata local nesses casos. **Objetivo**: O objetivo deste estudo foi avaliar parâmetros da imunidade inata e atividade de sialidases nos casos de VB em relação a maior ou menor participação das espécies de *A. vaginae*, *Leptotrichia* sp. e *Megasphaera* sp., além de comparar os níveis de citocinas pro-inflamatórias nos casos de VB de acordo com o *status* da infecção por CT. **Material e métodos:** Foram avaliadas mulheres que apresentaram VB no período do estudo e o grupo controle foi constituído por mulheres que apresentaram microbiota normal. Esfregaços vaginais foram utilizados para a classificação do padrão da microbiota vaginal. A quantificação das espécies de interesse foi realizada pela técnica de reação em cadeia da polimerase (PCR) em tempo real. A detecção de CTfoi realizada pela técnica de PCR qualitativo. A determinação da concentração das citocinas pró-inflamatórias Interleucina (IL)-1beta, IL-6, IL-8 e Fator de Necrose Tumoral (TNF)-alfa em

amostras de lavado vaginal foi determinada por ensaios imunoenzimáticos específicos. A atividade de sialidase presentes nessas amostras foi determinada através da conversão do substrato fluorogênico MUAN (2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid). **Resultados:** Os resultados da quantificação bacteriana apontam a associação das espécies avaliadas com a VB e para a heterogeneidade na produção de citocinas pró-inflamatórias e na atividade de sialidase presente entre os casos. Análises de componentes principais, considerando como parâmetros os níveis de IL-1β, IL-6, IL-8, TNFα, atividade de sialidase e quantidade de bactérias, mostraram distinção entre mulheres com microbiota vaginal normal e VB. O mesmo não pode ser observado através da comparação de mulheres com VB que apresentaram maior ou menor quantificação relativa de *A. vaginae*, *Leptotrichia* sp. e *Megasphaera* sp. Com relação à avaliação dos níveis de citocinas e a infecção clamidiana, mulheres com VB apresentaram aumento nos níveis de Il-1β, IL-6 e IL-8 na presença de CT, enquanto que os níveis dessas citocinas permanecem inalterados na microbiota vaginal normal, independentemente da presença de infecção por CT. **Conclusão:** Os resultados da comparação entre casos de VB quanto à maior ou menor participação de *A. vaginae*, *Leptotrichia* sp. e *Megasphaera* sp. na microbiota vaginal não apontaram para diferença entre os casos quanto aos parâmetros de resposta imune inata, quantidade de bacteriana e sialidase avaliados. Além disso, a resposta imune inata à VB é modulada pela presença de infecção clamidiana, que por sua vez, não apresenta influência na microbiota vaginal normal.

5. Abstract

Introduction: Bacterial vaginosis (BV) is the most frequent type of abnormal vaginal flora in women in childbearing age. Several gynecological and obstetrical complications are associated with BV, such as pelvic inflammatory disease, increased risk for acquisition of sexually transmitted infections (STIs), clinical and histological chorioamnionitis and low birth weight. Bacterial vaginosis is characterized by the replacement of the vaginal lactobacilli by other bacterial species, mostly anaerobes. Recent studies show that many species, rarely or never isolated by culture, are highly associated with BV, such as *Atopobium vaginae*, *Leptotrichia* sp. and *Megasphaera* sp. In common, these bacteria have the characteristic of producing lactic acid. Study of the vaginal flora of asymptomatic women showed that these species may replace the lactobacilli, dominating the vaginal environment. Thus, some authors suggest that *A. vaginae*, *Leptotrichia* sp. and *Megasphaera* sp. may contribute to a balanced vaginal flora. Considering that BV cases are microbiologically heterogeneous, the immune response is also likely to differ among the women. An imbalanced local immune response is one of the mechanisms leading to increased acquisition of STIs, such as chlamydial infection, which is very frequent in our population. Although studies demonstrated a significant association of BV with *Chlamydia trachomatis* (CT), few studies evaluated the associated innate immune response. **Objective**: The objective of this study was to evaluate parameters of the innate immunity and sialidase activity in BV, in relation to the larger or samaller participation of *A. vaginae*, *Leptotrichia* sp. and *Megasphaera* sp., and to compare the levels of pro-inflammatory cytokines in BV cases according to the status of CT infection. **Material and methods:** We evaluated women that presented BV in the period of the study and the control group was composed by women with normal vaginal flora pattern. Vaginal smears were used for the classification of the vaginal flora. The microorganisms of interest were quantified by real-time polymerase chain reaction (PCR). The detection of CT was performed by qualitative PCR. Vaginal rinsing samples were used to determine the levels of the pro-inflammatory cytokines Interleukin (IL)-1beta, IL-6, IL-8 and Tumor Necrosis Factor (TNF)-alpha by immunoenzymatic

assays. The sialidase activity was measured by the conversion of the fluorogenic substrate MUAN (2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid). **Results:** The results of the bacterial quantification indicate significant association of the tested species with BV and also a great heterogeneity of the pro-inflammatory cytokines and sialidase levels among the cases. Principal component analyzes, considering as parameters the levels of IL-1β, IL-6, IL-8, TNF-α, sialidase activity and bacterial amount, showed distinction between women with normal flora and BV. The same difference was not observed when comparing BV cases with larger or smaller relative amount of *A. vaginae*, *Leptotrichia* sp. and *Megasphaera* sp. In relation to the evaluation of the cytokine levels and CT infection, women with BV have increased levels of IL-1β, IL-6 e IL-8 in the presence of CT, while women with normal flora have unchanged levels of these cytokines, regardless of the CT status. **Conclusion:** The results of the comparison between BV cases with larger or smaller participation of *A. vaginae*, *Leptotrichia* sp. and *Megasphaera* sp. do not indicate differences among these cases in relation to characteristics of the innate immune response, bacterial amount and sialidase activity. Additionally, the innate immune response to BV is modulated by the presence of chlamydial infection, which does not interfere in normal vaginal flora.

Do *Atopobium vaginae, Megasphaera* sp. and *Leptotrichia* sp. change the local innate immune response and sialidase activity in bacterial vaginosis?

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[*] **Apresentado de acordo com as normas para submissão no periódico American Journal of Obstetrics and Gynecology.**

Abstract

Objective: To investigate if the participation of *Atopobium vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. in the vaginal community of bacterial vaginosis (BV) is associated with different pattern of innate immune response and sialidase activity.

Study design: This was a prospective, case-control study that included vaginal samples from 205 women with BV and 205 women with normal flora. Pro-inflammatory cytokine and sialidase levels, as well as absolute quantification of BV associated bacteria and total bacterial load were determined in all samples.

Results: Bacterial load, as well as level of interleukin 1-beta and sialidase activity are highly variable in BV and increased when compared to normal flora. BV cases with larger amounts of *Atopobium vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. do not present different patterns of antibacterial response.

Conclusion: Presence of high amounts of *A. vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. in BV-associated flora does not implies in an altered innate immune response and sialidase activity.

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Key words:*Atopobium vaginae*, bacterial vaginosis,innate immunity, sialidase.

Introduction

Bacterial vaginosis (BV) is a condition defined as an alteration on the vaginal flora.[1] Although the etiology of BV is unclear, it can be described as vaginal flora with decreased or absent of *Lactobacillus* spp., with an overgrowth of anaerobic bacteria (such as *Gardnerella vaginalis, Mycoplasma hominis, Prevotella* spp., *Mobiluncus* spp, and *Bacteroides fragilis*).[1,2] These anaerobes produce a great variety of microbial products that can interfere in the host´s inflammatory response, such as sialidase that can degrade secreted IgA.[3] Sialidase-producing *G. vaginalis* strains have higher adherence capacity to epithelial cells and biofilm formation[4] and women with BV and high sialidase levels in early-pregnancy are in increased risk for preterm birth.[5]

Considering the high heterogeneity in the microbial composition of BV, it is expected that the inflammatory response may vary according to the pathogens found in each case. The inflammatory response to BV is characterized by increased Interleukin (IL)-1β and unchanged IL-6 and TNF-α levels, while few divergences remain regarding the IL-8 levels.[6-9] In fact, pro-inflammatory cytokine release in BV is one of the proposed mechanisms for increasing risk for sexually transmitted infections (STIs) acquisition, such as HIV.[10] Thus, it would be reasonable to propose that the microbial composition of BV could determine which BV cases are more or less deleterious to women's health.

More recently, molecular-based investigations have added more information regarding the BV associated bacteria, by linking other species to this condition such as *Atopobium vaginae,* BV associated bacteria (BVAB1-3), *Megasphaera* sp. and *Leptotrichia* sp.[11,12] In a cultivation-independent study, Zhou et al.[13] showed that, in fact, asymptomatic women may have a vaginal microbiota dominated by the *A.*

vaginae,Megasphaera sp. and *Leptotrichia* sp., and support the idea that although they are associated to BV, they also have the capability to produce lactic acid. Based on that, authors[14-16] have proposed that the replacement of vaginal lactobacilli by *A. vaginae,Megasphaera* sp. and *Leptotrichia* sp. should not be considered as abnormal, since these species would be sufficient for establishing a healthy environment by lowering the local pH.

The observation of some vaginal flora dominated by lactic acid producers, other than lactobacilli in normal asymptomatic women[13] raises the hypothesis that a larger participation of the species would not be deleterious forthevaginal environment. Therefore we aimed to verify if the participation of *A. vaginae,Megasphaera* sp. and *Leptotrichia* sp. in BV-associated flora defines differences on the pro-inflammatory cytokine profile, sialidase activity and bacterial load in BV.

Material and methods

Subjects

This was a prospective case-control study, which included premenopausal, non-pregnant women attending an outpatient clinic of the "Centro de Atenção Integral a Saúde da Mulher (CAISM)", at Campinas University, Campinas, Brazil and one unity of primary medical care in Botucatu, Brazil from May 2009 to October 2011. Minimum sample size of 200 women for each group was calculated based on the estimated variability on the level of IL-1β and IL-8 assuming α=0.05 and β=0.20.

All women seen at routine of both services presenting full condition for inclusion at time were candidates for enrollment. We excluded women who presented

vaginal bleeding, urinary loss, use of systemic antibiotic or vaginal medication during the last 30 days or reported that had sexual intercourse or any vaginal procedure in the preceding 72 hours.

Before the standard pelvic examination, all women were explained about the aim of the study and signed a consent term. They answered a specific designed form for collection of demographic, behavioral and clinical data. All information obtained were based on self-reports. In case of doubt or discomfort to answer the interview, women were asked to skip the question with no disadvantage to their inclusion in the study. This study was approved by the Ethics Research Committee from Campinas University (Protocol 282/2009) and Botucatu Medical School (Protocol 2936/2008).

Sampling procedures

Following the standard routine of both services, all women undergoing examination, after having an unmoistened sterile speculum inserted, were checked for vaginal pH by pressing strips with range of 4.0-7.0 (Merck, Darmstadt, Germany) against the vaginal wall and comparing the final color with the scale provided. Whiff test was performed by adding two drops of a 10% KOH in a cotton swab saturated with vaginal content and results were interpreted by the practitioner as positive, doubtful or negative. For microscopic evaluation of the vaginal flora, one cotton swab was sampled from the mid-lateral vaginal wall. Smears were immediately confectioned in two glass slides. One slide was evaluated by wet mount microscopy for detection of *Trichomonas vaginalis* and *Candida* sp. morphotypes. The remaining smear was stained by Gram's method and used for vaginal flora classification according to Nugent's scoring system.[17] Cervical samples were also taken by rotating the

endocervical canal 3 times 360° with a cytobrush for assessment of presence of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) cervicitis by PCR.[18,19]

Additional sampling step consisted of a vaginal rinsing with 3 mL of sterile 0.9% NaCl using a sterile plastic pipette, allowing the contact of the solution with the lateral vaginal wall. Professionals in charge of this procedure were trained on how to perform the rinsing and oriented to recover exactly 3 mL of the solution. If lower volumes were recovered or if blood was visually detected in the samples, they were immediately discarded. Samples were centrifuged at 800 x *g* for 10 minutes. Supernatants were used on cytokine and sialidase assays, while DNA for bacterial quantification was obtained from pellet samples. Both, supernatants and pellets were stored at -80^{o}C until analysis.

Criteria for selection of subjects for analysis

From 944 women, we included a total of 313 (33.2%) women with confirmed diagnosis of BV by microscopy (Nugent's score ranging from 7 to 10). We excluded cases of BV PCR-positive forCT (n=74, 23.6%), NG(n=2, 0.6%) and with both (n=4, 1.3%). Samples of vaginal rinsing that presented positive *T. vaginalis* resultin the analysis by wet mount microscopy were discarded immediately after sampling. Additionally, women in which *Candida* sp. morphotypes (n=28, 7.3%) were detected on the vaginal smears were also excluded from the study, independently if clinical sign of candidosis were present or not. With this, we assured that all the remaining 205 BV samples evaluated in the study consisted of BV cases with a minimal chance of current co-infection. In order to constitute the control group, we included randomly 205

women with normal flora (Nugent's core from 0 to 3) that did not present any concurrent genital infection.

Quantitative PCR (qPCR)

We quantified 4 BV associated bacteria (*G. vaginalis,A. vaginae, Megasphaera* sp., and *Leptotrichia* sp.) and the total bacterial load (through the amplification of a conserved region of 16S rRNA gene) in women with BV and normal flora. Total DNA was extracted from the pellets using QIAamp DNA Mini Kit (Qiagen,Valencia, CA) according to manufacturer's protocol for Gram-negative and positive bacteria. Final elution step was performed in 100μL of the provided buffer and volumes of 2μL were used as the qPCR template. Taxon-directed and broad range 16S rRNA reactions were performed individually in a 13 μL final volume with Maxima SYBR Green/ROX (Fermentas, St. Leon-Rot, Germany) with the couple of primers in a concentration of 0.3μM each (Table 1). Assays were run on the LineGeneK (Bioer, China) in 40 cycles of amplification.

Bacterial load from the unknown samples were calculated through the interpolation of their cycle threshold (CT) from a standard curve constituted by 10-fold serial dilution of the plasmids DNA. To obtain the plasmids, we amplified bacterial sequences of *A. vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. from clinical samples and from pure cultures of *G. vaginalis* (ATCC 14018) and, for the conserved 16SrRNA region, from *Staphylococcus aureus* (ATCC 19095). Amplicon size was confirmed by electrophosis on 1.5% agarose and bands were excised from the gel. After bands purification, products were sequenced in Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Inc). Final sequences were submitted to analysis using BLAST

algorithm (available at www.ncbi.nlm.nih.gov) to confirm they matched to the sequences of interest. Amplified sequences were cloned into *Escherichia coli* DH5-α (Invitrogen, Carlsbad, CA) using the Kit pGEM T Easy Vector System (Promega, Madison, WI). After *E. coli* transformed cells, plasmid DNA was extracted using the Wizard Plus Minipreps (Promega, Madison, WI). Extracted plasmid DNA was quantified and the referent number of copies/μL was calculated using Avogado's equation.

Every sample was run in duplicate and if difference superior to 1 cycle threshold between the two values were observed, sample was retested. Mean values for each sample were calculated and data were expressed by number of copies/mL of vaginal rinsing. Final bacterial amounts were obtained by multiplying the mean value obtained from the 2μL of template used in each reaction by 50 in order to reach the elution volume of 100μL that corresponded to 1mL of vaginal rinsing.

Determination of the relative amount of A. vaginae, Megasphaera *sp*. and Leptotrichia *sp*.

Relative amount of *A. vaginae, Megasphaera* sp. and *Leptotrichia* sp. in BV samples was calculated by the sum of the absolute quantification result of all three microorganisms and subsequently dividing it by the total bacterial DNA present in the sample, estimated by result of the qPCR for the broad range 16S rRNA. Median of the final relative amounts was calculated to determine the cut-off to separate samples according to the small or large participation these species in the total bacterial core of BV.

Measurement of cytokine levels

Interleukin-1β IL-6, IL-8 and TNF-α levels were measured in the supernatants vaginal rinsing by ELISA (Duo Set Kits, R&D Systems, Minneapolis, MN). All the 205 BV and 205 normal flora samples were tested in duplicates and final concentration was determined by their mean value. If concentration values obtained were set above the standard curve range, the sample was diluted (1:5 and 1:10) and retested. The mean intra-assay variability was 7.4% for IL-1β, 9.8% for IL-6, 9.1% for IL-8 and 23.4% for TNF-α. Inter-assay variability rates were 4.1% for IL-1β, 5.3% for IL-6, 5.1% for IL-8 and 25.5% for TNF-α. The minimum detectable levels for IL-1β, IL-6, IL-8 and TNF-α assays were, respectively, 0.2 pg/mL, 3.2 pg/mL, 20.0 pg/mL and 1.1 pg/mL.

Sialidase assay

Measurement of sialidase activity in the supernatants was performed using the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN; Sigma-Aldrich, St. Louis MO). In a 96-well plate (OptiPlate-96F, PerkinElmer, Waltham MA), samples were added to 50μL of 0.35% MUAN (wt/vol) and incubated for 30 minutes at 37^{o}C. The reactions were read at 450 nm, 365 nm excitation and filter at 420 nm in spectrofluorometer Epoch (with Gene5 software; Biotek, Winooski VT). Unknown values were calculated from the standard curve of 10-fold dilution of purified *Clostridium perfringens* neuroaminidase (Sigma-Aldrich, St. Louis MO), ranging from 1000.0 to 1.0 ng/mL. All samples were tested in duplicate. In each assay a negative control consisting of a sample previously heated at 95^{o}C for 15 min and a positive control of a known sialidase-positive sample with visible fluorescence on UV transilluminator were run together with the unknown samples.

Statistics

Comparison of discrete and continuous variables of the two study groups were performed respectively by Chi-squared and the non-parametric Mann-Whitney test. Bacterial load, cytokine and sialidase levels between the groups were compared using Mann-Whitney test. Correlation between the species was evaluated by the Spearman correlation test. Analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) and $P<0.05$ was considered as significant.

Principal component analysis (PCA) is tool by which complex data are converted in simple values (principal components), allowing to evaluate the samples based on the different variables they present. We performed this analysis to compare normal flora with BV samples, as well as to compare BV cases with small or large amounts of *A. vaginae, Megasphaera* sp. and *Leptotrichia* sp, based on the combination of their cytokine levels, sialidase activity and bacterial load. We performed the auto-scaled PCA analysis with $\log_{10}$ transformed data using MVSP software, version 3.13a (Kovach Computing Services, Pentraeth, Wales, UK).

Results

Population characteristics

Demographic, behavioral and gynecologic characteristics of the enrolled women with BV and normal flora are shown in Table 2. Population was homogeneous distributed in the two study groups, not differing at any of the demographic or behavioral parameters except for smoking habit that was more frequent among BV (p=0.01). Regarding gynecologic data, women with BV were more likely to had presented at least one previous episode of BV than the control group (p=<0.0001).

Larger number of nulliparous was found among women with normal flora (p=0.003). As expected, vaginal pH was significant higher in BV women (p<0.0001) and number of samples with positive or doubtful whiff test were more frequent in BV group (<0.0001).

Amounts of BV associated bacteria and total bacteria

The results of taxon-directed qPCR showed that all tested BVassociated bacteria were detected not only in BV samples, but also in the control group (Table 3). The species with strongest association with BV was *A. vaginae*, detected in 97.6% of the samples; although it was also detected in 73 (35.6%) women with normal flora. A slight inferior rate was shown by *G. vaginalis,* which was present in 93.2% of BV and in 22.9% of normal samples. *Leptotrichia* sp. had the lowest rate of detection in BV, even though it was present in 63.0% of the samples. Regarding the vaginal quantity of each individual species, all showed significant larger amounts in BV when compared to normal flora (P<0.0001). Quantification of the broad range 16S rRNA gene reveled that the total bacterial amount in the vagina of women with normal flora is highly variable (ranging from 10^4 to 10^{11}) but still significantly lower than in BV.

Association among the BV associated bacteria

As shown in Table 4, there is an important association among the BV associated bacteria in women with BV. The strongest association observed was between *A. vaginae* and *G. vaginalis* that were detected simultaneously in 91.7% of the BV samples. The combination between *G. vaginalis* and *Leptotrichia* sp. showed the lowest association rate, but was still present in more than half of the BV samples (61.0%). Regarding the vaginal load of the tested BV associated bacteria, analyzes

showed a significant positive correlation for all species, except for the combination of *G. vaginalis* and *Leptotrichia* sp. (Table 4). Although of Spearman correlation ranks varied among the different combinations of species, all of them showed a positive correlation rate.

Vaginal levels of cytokines and sialidase in BV and normal flora

Interleukin 1-β had detectable levels in 81 (39.5%) and 178 (86.8%) vaginal samples from women with normal flora and BV, respectively. As observed in Figure 1, IL-1β concentrations were significantly higher in women with BV (median 63.5pg/mL; range 0.0-1950.0) when compared to normal flora (median 4.7pg/mL; range 0.0-320.0, P<0.001). Levels of IL-6 were below the detection limit in most of normal (n=159, 77.6%) and BV samples (n=132, 64.4%) and did not differ in concentration between the groups (P>0.05). Women with BV and normal flora had both IL-6 median of 0.0pg/mL; ranging from 0.0 to 258.3 in normal and from 0.0 to 324.4 in BV. Concerning IL-8, most of the samples had detected levels; 183 normal women (89.2%) and 184 BV (89.7%) women. No significant change in IL-8 level was found in BV (median 245.1pg/mL; range 0.0-2220.0) when compared to normal flora (median 231.4 pg/mL; range 0.0-2010.0); (P>0.05). Few samples had detectable levels of TNF-α, 30 (14.6%) and 29 (14.1%) from normal and BV group, respectively. Levels of TNF-α remained unchanged in BV (median 0.0pg/mL; range 0.0-142.2) in relation to normal flora (median 0.0pg/mL; range 0.0-657.9); (p>0.05). Sialidase activity was detected in 128 (62.4%) women from BV groups while only four out of 205 women with normal flora showed measurable activity. Women with BV had increased sialidase activity when compared to control group.

Principal component analysis (PCA)

We first used the PCA method to evaluate the distribution of the groups with normal flora and with BV according to all measured parameters. As shown in Figure 2A, a clear division in two groups can be observed, with the predominance of the normal flora group on the left side of the graph. Although few BV samples are found overlapping the graph area where normal cases predominate, there is an evident division between these two groups. The contribuiting rates for PC1 and PC2 were, respectively, 52.9 and 15.0%. In the second PCA (Figure 2B), we aimed to verify if BV cases with large relative amounts of *A. vaginae, Megasphaera* sp. e *Leptotrichia* sp. would differ from cases in which other bacteria predominate. In this PCA the results of the qPCR for *A. vaginae, Megasphaera* sp. e *Leptotrichia* sp. and broad range 16S rRNA were not included as this parameters had been previously used for establishing the two groups. As shown in Figure 2B, samples from the group with small and large load of lactic acid-producing bacteria were randomly distributed on the graph area. In this analysis, PC1 and PC2 accounted for 43.5% and 27.8% of variability.

Comment

In this study we could determine the profile of pro-inflammatory cytokines, sialidase, number total bacteria and BV-associated microorganisms, including *A. vaginae, Megasphaera* sp.and *Leptotrichia* sp. in vaginal samples from women with BV and normal flora. Our inclusion criteria were very strict to assure that none of the BV or normal samples had concurrent asymptomatic cervicitis, candidosis or trichomoniasis. In fact, we excluded from our analyses 80 (25.6%) BV samples because

they were PCR-positive for CT and/or NG. The present findings are in agreement with the well documented association of BV and CT and NG infection[23], which hinder the execution of studies aiming the analysis of cases of BV solely.

The studied population was homogeneous between the groups, not differing in most of the evaluated characteristics. Although important studies showed that BV is associated with ethnicity, number of sex partners and education level,[24,25] we failed to demonstrate these associations. This might be due to the fact that this study was not designed aiming to search for BV-associated factors or even because demographic and behavioral data were collected by interview and despite our efforts to create ideal conditions for an accurate answer, they could still be imprecise. On the other hand, as this research was not focused on epidemiology, and hence we had no hypothesis to be confirmed the responses on the relationship of BV with sexual habits and BV may be less biased. As we have excluded almost 1 of 3 of the women due to STD like CT and NG, this may explain why sexual history was no longer associated with BV. As a consequence, future epidemiologic studies on sexual aspects of BV should only be performed if all other STIs are meticulously excluded. We found that women with BV were more likely to smoke regularly and had been pregnant at least once, what is in agreement with previous findings of association between BV and number of living births and smoking habit.[25,26] As expected, BV women had significant higher vaginal pH and were more likely to have positive Whiff test since both are considered important markers for BV. [27]

In relation to the results of the quantification of BV-associated bacteria, it can be noted that BV-associated bacteria are not only detected more frequently, but they

are also found in significant larger amounts in the vagina of BV women. Several reports already suggested the potential use of quantitative methods as reliable tools for diagnosing BV, by combining the absolute number of some BV-associated bacteria in vaginal flora.[21,28,29] In addition, our findings showed that the total bacterial load in normal vaginal flora is highly variable but also significantly lower than the bacterial amount in BV. Furthermore, our qPCR data showed a significant increase on total number of bacteria copies through the amplification of 16S rRNA gene. Although it was already demonstrated the increase in bacterial number in BV[30,31] this study adds the information regarding the estimated number of copies present in each sample. We are aware of this technique's limitation, such as difference in efficiency of the reaction among the different species. However we consider this approach more reliable in determining the total bacterial load from vaginal samples than the simple quantification of the extracted DNA that may carry large amounts of host's DNA from epithelial and inflammatory cells.

Another important finding here presented is the important positive correlation that exists among all the evaluated BV-associated species except between *G. vaginalis* and *Leptotrichia* sp. Our analysis adds to the previous information that the BV associated microorganisms are found simultaneously in vagina,[13,20,32] the fact that their growth seems to be positively correlated, supporting the idea of a probable synergism among the most important BV-associated bacteria. Recently, Menard et al.[29] showed that combination of absolute quantification of *G. vaginalis* and *A. vaginae*, above 10^9 and 10^8 number of copies/mL respectively, is a sensible and specific tool for the diagnosis of BV. Actually, a positive correlation rate between *G. vaginalis* and *A. vaginae* number in BV was previously reported by DeBacker et al.,[33] but here we

provide the new information regarding the correlation rates among the other BV species which may provide new ideas for further researches for implementation of a routine application of qPCR for detecting BV.

In addition to bacterial quantification, we further assessed pro-inflammatory cytokine levels and sialidase activity as parameters to detect possible variations among the BV samples. As BV is a polymicrobial entity and bacterial composition varies among the cases, differences in the inflammatory response and microbial products are expected. Our findings regarding cytokine levels in BV are in agreement with the literature that shows increased IL-1β and unchanged IL-6, IL-8 and TNF-α levels in BV when compared to normal flora.[6-8] Concerning sialidase activity, we confirmed that it is almost exclusively detected in BV flora, as recently reported.[3] Additionally, levels of activity of this enzyme in BV are extremely variable reflecting the diversity on bacterial species and also the different virulence potential among the strains composing the vaginal flora.[4,34]

In view of the high diversity on bacterial load, sialidase and cytokine levels on vaginal samples, we considered these measures as good parameters for analyzes aiming to group samples based to their similarity. In the first PCA analysis, we compared BV and normal flora samples aiming to (1) validate this method of analysis for grouping vaginal samples based on the described parameters, which was confirmed by the clear separation on the distribution of BV and normal samples; (2) check this method of representation would reflect the heterogeneous feature of BV, which could be observed in the spread distribution of BV samples on the graph, with more or less proximity to the normal group, which tended to be more concentrated. In the same

way, Yoshimura et al.[32] successfully employed the same analysis to group vaginal samples according to their bacterial composition, demonstrating the usefulness of this method to analyze samples using complex data.

Accordingly, we separated our BV samples in two groups based on the relative number of lactic acid-producing bacteria *A. vaginae, Megasphaera* sp. and *Leptotrichia* sp., in order to verify if a greater participation of these species in the composition of BV-associated flora establishes differences in patterns among BV samples. Unlike the clear division between BV and normal flora, no tendency on division was observed between samples with smaller or larger relative amounts of *A. vaginae, Megasphaera* sp. and *Leptotrichia* sp. Results from this second PCA show that samples with differences in the number of these bacteria present a substantial similarity on the innate inflammatory response, as well as on the sialidase activity.

The results here presented do not corroborate with the assumption that the higher participation of *A. vaginae, Megasphaera* sp. and *Leptotrichia* sp. on the vaginal flora is associated with a healthy vaginal environment.

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Table 1. Primer sequences used in the quantitative PCR.

Target	Primer name	Primer sequence	Amplicon size
Atopobium vaginae	Atop582F	5'- TAGGCGGTYTGTTAGGTCAGGA-3'	81bp[20]
	Atop665R	5'- CCTACCAGACTCAAGCCTGC-3'	
Megasphaera sp.	MegaE-456F	5'- GATGCCAACAGTATCCGTCCG-3'	211bp[20]
	MegaE-667R	5'-CCTCTCCGACACTCAAGTTCGA-3'	
Leptotrichia sp.	Lepto-395F	5'CAATTCTGTGTGTGTGAAGAAG-3'	251bp[20]
	Lepto-646R	5'-ACAGTTTTGTAGGCAAGCCTAT-3'	
Gardnerella vaginais	GV1F	5'-TTACTGGTGTATCACTGTAAGG-3'	332bp[21]
	GV3R	5'-CCGTCACAGGCTGAACAGT-3'	
Broad range 16S rRNA	Eub341F	5'-CCTACGGGAGGCAGCAG-3'	193bp[22]
	Eub534R	5'-ATTACCGCGGCTGCTGGC-3'	

Table 2. Demographic, behavioral and gynecological characteristics from 250 women with normal flora and 205 with BV included in the study.

Subjects characteristics	Normal flora	Bacterial vaginosis	*P*
Age, median (range), years	**29 (18-50)**	**32 (18-52)**	**0.64**[a]
Race (self-defined), n (%)			
White	110/154 (71.4)	115/185 (62.2)	
Nonwhite	44/154 (28.6)	70/185 (37.8)	0.07[b]
Marital status, n (%)			
Single	49/181 (27.1)	68/199 (34.2)	
Married	132/181 (72.9)	131/199 (65.8)	0.13[b]
Years at school, median (range)	**11 (1-18)**	**9 (1-16)**	**0.06**[a]
Remunerated activity, n (%)	**108/182 (59.3)**	**116/195 (59.5)**	**0.97**[b]
Smoking habit, n (%)	**33/185 (17.8)**	**56/198 (28.3)**	***0.01***[b]
Sex partners, prior 12 months, n (%)			
0 or 1	169/182 (92.9)	177/199 (88.9)	
2 or more	13/182 (7.1)	22/199 (11.1)	0.19[b]
Number of vaginal intercourse/week, median (range)	**2 (0-7)**	**2 (0-7)**	**0.98**[a]
Previous BV, n (%)	**35/173 (20.2)**	**70/159 (44.0)**	***<0.0001***[b]
Previous STD, n (%)	**25/171 (14.6)**	**17/183 (9.3)**	**0.12**[b]
Consistent condom use, n (%)	**15/164 (9.1)**	**25/185 (13.5)**	**0.20**[b]
Hormonal contraception use, prior 12 months, n (%)	**35/164 (26.8)**	**43/185 (23.2)**	**0.67**[b]
Parity, n (%)			
0	74/182 (40.7)	59/198 (29.8)	
≥1	108/182 (59.3)	139/198 (70.2)	*0.003*[b]
Vaginal pH, median (range)	**4.4 (4.0-5.0)**	**5.0 (4.0-7.0)**	***<0.0001***[a]
Whiff test, n (%)			
Positive or doubtful	20/177 (11.3)	134/187 (71.7)	
Negative	157/177 (88.7)	53/187 (28.3)	***<0.0001***[b]

Total of women may vary among the categories, as some of the data were unavailable or due to patient´s refusal to answer;
[a]Mann-Whitney non parametric test;
[b]Chi-squared test.

Table 3. Bacterial vaginosis associated bacteria and total bacterial load in women with normal flora and BV. Number of positive cases for each species (N) and median bacterial load (number of copies/mL) in the vaginal samples.

	Normal (N=205)		Bacterial vaginosis (N=205)		
Species	**N (%)**	**Number of copies/mL; Median (range)**	**N (%)**	**Number of copies/mL; Median (range)**	**P***
Gardnerella vaginalis	47 (22.9)	0.0 (0.0, 8.3E+07)	191 (93.2)	3.5E+06 (0.0, 9.5E+08)	<0.0001
Atopobium vaginae	73 (35.6)	0.0 (0.0, 3.0E+07)	200 (97.6)	9.6E+08 (0.0, 4.3E+10)	<0.0001
Megasphaera sp.	11 (5.4)	0.0 (0.0, 3.3E+05)	188 (91.7)	2.7E+08 (0.0, 1.8E+10)	<0.0001
Leptotrichia sp.	16 (7.8)	0.0 (0.0, 4.3E+05)	129 (63.0)	2.0E+07 (0.0, 5.7E+10)	<0.0001
16S rRNA	205 (100.0)	1.9E+09 (1.0E+04, 1.0E+11)	205 (100.0)	1.0E+10 (1.0E+08, 3.3E+11)	<0.0001

*Mann-Whitney test.

Table 4. Number of cases with coexisting species (N) and correlation rank (r) among the 205 women with bacterial vaginosis.

	G. vaginalis **(N=191)**		***A. vaginae*** **(N=200)**		***Megasphaera*** **sp.(N=188)**		***Leptotrichia*** **sp. (N=129)**	
Coexisting species	**N (%)**	**r**	**N (%)**	**r**	**N (%)**	**r**	**N (%)**	**r**
Gardnerella vaginalis	--	--	188 (91.7)	0.4**	175 (85.0)	0.16*	125 (61.0)	0.06
Atopobium vaginae	188 (91.7)	0.40**	--	--	184 (89.8)	0.66**	128 (62.5)	0.46**
Megasphaera sp.	175 (85.0)	0.16*	184 (89.8)	0.66**	--	--	127 (62.0)	0.57**
Leptotricha sp.	125 (61.0)	0.06	128 (62.5)	0.46**	127 (62.0)	0.57**	--	--

*p=0.02; **p<0.0001.

Figure 1. Vaginal levels of pro-inflammatory cytokines and sialidase in women with bacterial vaginosis (n=205) and normal flora (n=205).

Figure 2. Scatter plots of the principal component analysis (PCA) case scores parameters of (A) women with bacterial vaginosis and normal flora and (B) bacterial vaginosis cases with low and high relative amount of the lactic acid-producers (*A. vaginae*, *Megasphaera* sp. and *Leptotrichia* sp.), using the median as the cutoff point. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

Figure 1.

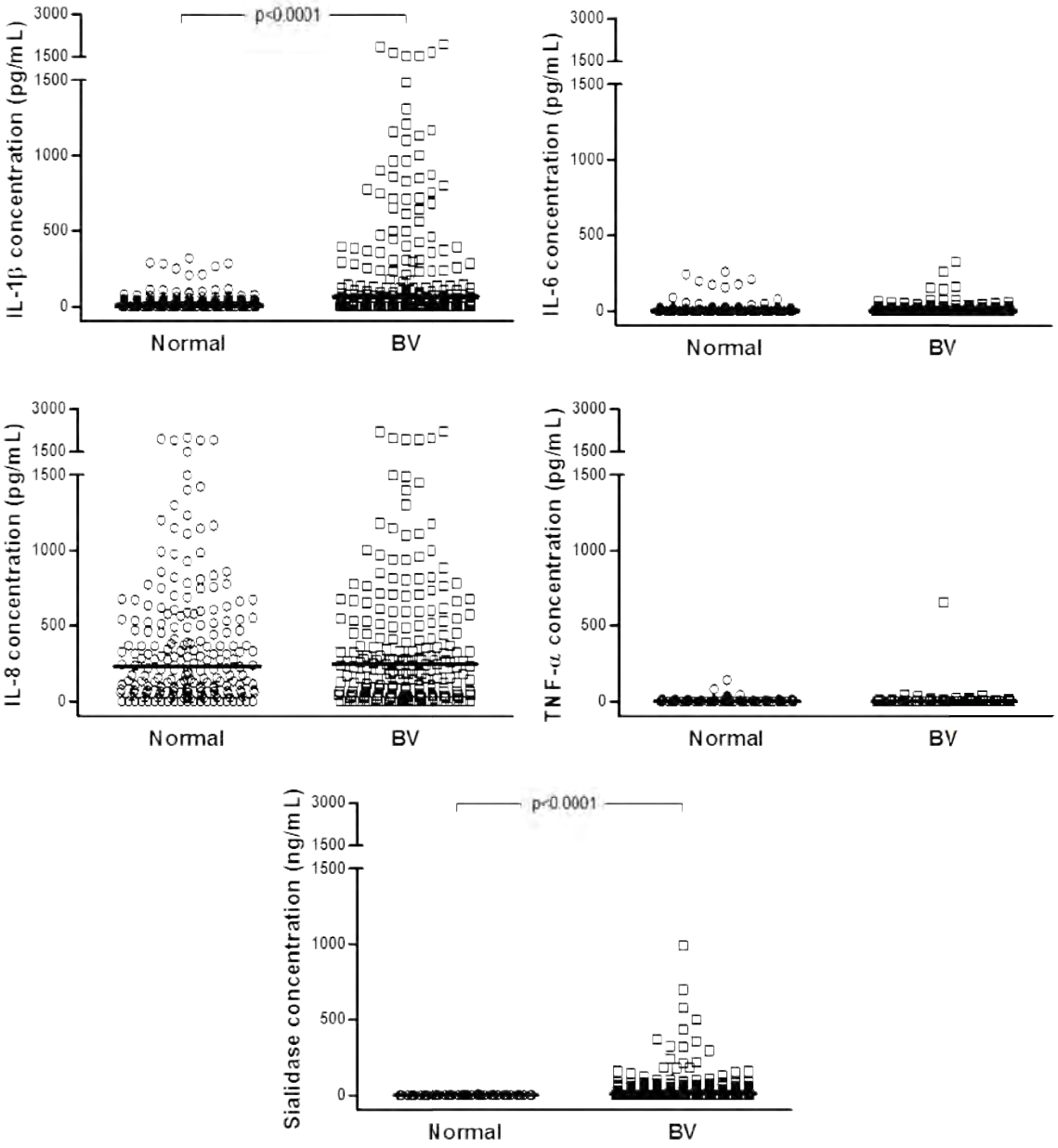
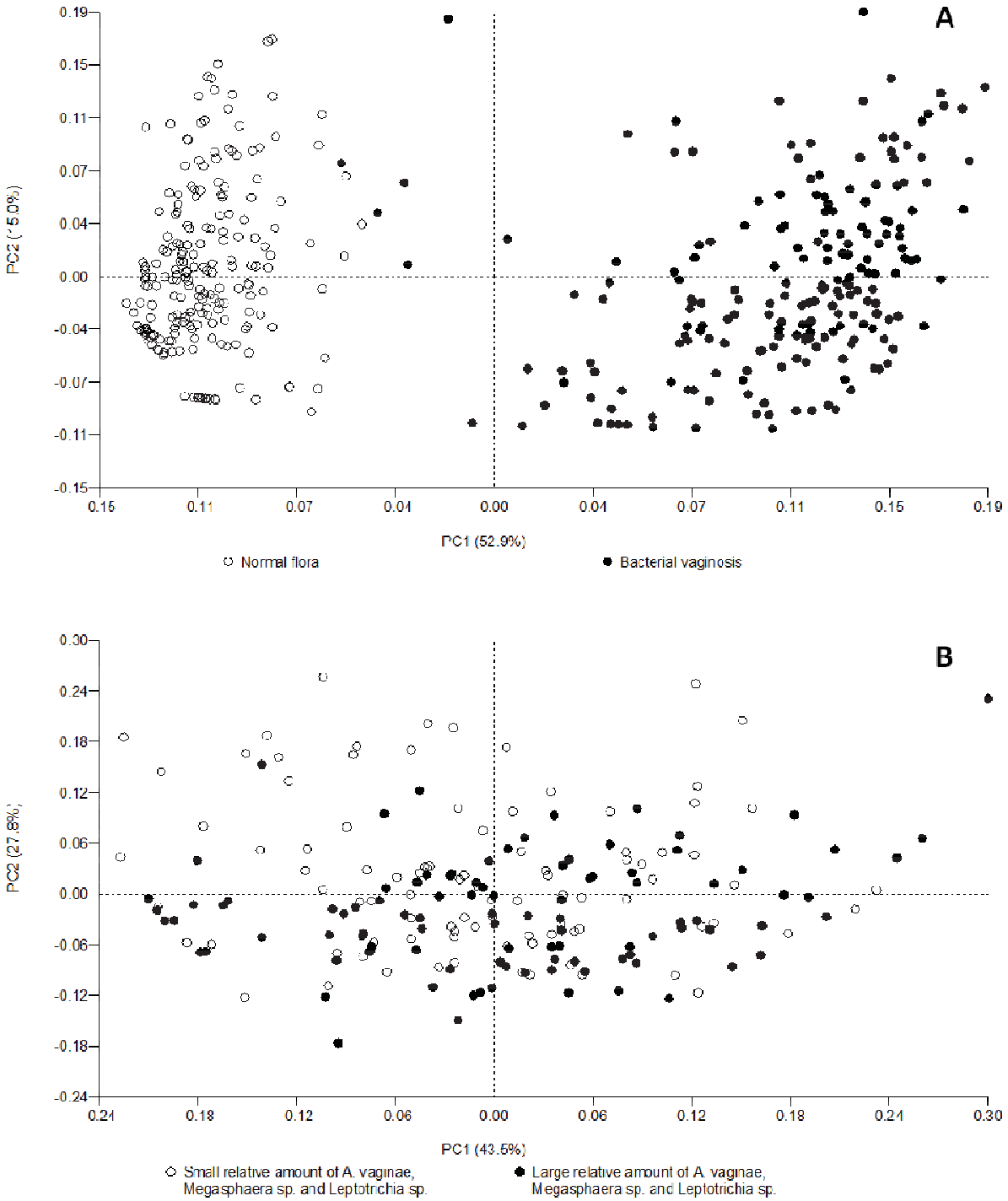

p<0.0001
IL-1β concentration (pg/mL)
3000
1500
1500
1000
500
0
Normal
BV
IL-6 concentration (pg/mL)
IL-8 concentration (pg/mL)
TNF-α concentration (pg/mL)
p<0.0001
Sialidase concentration (ng/mL)

Figure 2.


A
PC2 (15.0%)
PC1 (52.9%)
Normal flora
Bacterial vaginosis
B
PC2 (27.8%)
PC1 (43.5%)
Small relative amount of A. vaginae, Megasphaera sp. and Leptotrichia sp.
Large relative amount of A. vaginae, Megasphaera sp. and Leptotrichia sp.

Changes in the innate immune response due to chlamydial infection in the presence of bacterial vaginosis

Running title: Vaginal cytokines and chlamydial infection.

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Precis: Innateimmune response to bacterial vaginosis is increased in the presence of chlamydial infection, but remains unchanged in normal flora.

Abstract

Objective: We aimed to verify if the innate immune response to BV and normal flora is modulated by CT infection, through the assessment of vaginal pro-inflammatory cytokines.

Material and methods: In this prospective, case-control study, we evaluated vaginal samples from women with normal flora and BV in relation to the levels of the pro-inflammatory cytokines Interleukin (IL)-1 beta, IL-6, IL-8 and tumor necrosis factor (TNF)-α, by enzyme-linked immunoabsorbent assay (ELISA). Study groups were composed considering the PCR result for CT infection. From 256 women with BV detected during the period of the study, 68 (26.7%) tested positive for CT while 188 (73.4%) women were negative. As control groups, we randomly selected 68 with CT infection and 188 negative women with normal flora that were sampled in the same period. Cytokine levels between the groups were compared by Mann-Whitney test, $P<0.05$.

Results: We found that BV was associated with increased in IL-1β ($P<0.001$) and unchanged IL-6, IL8 TNF-α, when compared to normal flora. Presence of CT did not change vaginal levels of any of the tested cytokines in normal flora. Among women with BV, levels of IL-1β ($P=0.02$), IL-6 ($P=0.02$), IL-8 ($P=0.03$) were elevated in the presence of CT cervicitis.

Conclusion: Presence of CT infection modulates the vaginal innate immune response to BV, but do not alter cytokine levels in normal flora.

Key words: Bacterial vaginosis, chlamydial infection, innate immunity, cytokines.

Introduction

Bacterial vaginosis (BV) is the most common type of altered vaginal flora.[1] Its prevalence is nearly 30% in women in reproductive age, being asymptomatic in 50% of the cases.[2] The etiology of BV is polymicrobial, since it is characterized by the replacement of vaginal lactobacilli by other bacterial species, mostly anaerobes.[3] Cultivation-independent studies recently demonstrated an increasing number of BV-associated bacteria and the high diversity in the species in the different cases of BV.[4,5] Due to its heterogeneous microbial feature, immune response is expected to be variable among women experiencing BV.

Although by definition BV is not considered an inflammatory condition, studies are concordant in showing that it is associated with increased levels of local interleukin (IL)-1beta.[6-8] High levels of IL-1β are not followed by IL-6 and IL-8 in BV, as evidenced by unchanged numbers of leukocyte on vaginal smears when compared to normal vaginal flora.[7,8] Cytokine production in response BV is mainly triggered by bacterial components and products that activate toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors present in epithelial cells from the vaginal surface or in resident macrophages, constituting the first line of defense against BV.[9]

Several studies showed that BV is considered an important risk factor for several sexually transmitted infections (STIs), such as human immunodeficiency virus (HIV) infection, gonorrhea, herpes, trichomoniasis, and chlamydial infection.[10-14] Recent study showed that not only BV facilitates *Chlamydia trachonatis* (CT) acquisition, but also CT contributes for development of BV flora, independent of other known risk factors.[10] Several studies have demonstrated mechanisms that could facilitate STIs acquisition in the presence of BV, and they all point to the imbalance of the innate immune response cause triggered by BV. *C. trachomatis* is the most frequent bacterial sexually transmitted disease in the United States[15] and found in high rates of our population.[16,17] Similarly to BV, first defense against CT infection

is played by local macrophages and epithelial cells, through the activation of TLRs and NODs receptors with production of the pro-inflammatory cytokines, but from endocervical region.[18]

Until now, there are few reports in the literature describing the vaginal levels of pro-inflammatory cytokines in BV with CT infection and they are restricted to a small sample size or did not evaluated the effects of CT alone in VB.[19,20] In fact, several sampling procedures can be used on studies assessing cytokine levels cytokine on lower genital tract, but recent work has demonstrated that cervicovaginal lavage with physiologic pH solution is a reliable sampling method for this type of approach.[21] Therefore, our aim was to verify if the innate immune response to BV and normal flora is altered in the presence of CT infection, through the assessment of pro-inflammatory cytokines in vaginal samples.

Material and methods

In a two-years period, from 2009 to 2011, we enrolled women attending gynecology clinics from Botucatu Medical School, at São Paulo State University, and units of primary health care in Botucatu, Brazil, and from the "Centro de Atenção Integral a Saúde da Mulher (CAISM)", at Campinas University, Campinas, Brazil. Only non-pregnant women and in childbearing age were invited to participate.

The composition of the groups for analysis was performed according to the total cases of BV detected by microscopy in the period of the study, excluding the cases in which BV co-existed with candidosis or aerobic vaginitis. From the total of 267 BV cases detected among women included in the study, 11 (4.1%) were excluded as they tested positive for NG. The remaining 256 BV cases were separated, according to the CT status, in 68 (26.6%) CT-positive and 188 CT-negative samples. We randomly chose from the women with normal flora and negative result for NG sampled in the same period, 68 cases with CT infection and 188 cases

with negative PCR for this infection to match the sample size of the groups with BV flora. Enrolled women provided a written consent term and this study was approved at the ethics committees from Campinas University (Protocol 282/2009) and Botucatu Medical School (Protocol 2936/2008). Women presenting vaginal bleeding, urinary loss, last sex intercourse in the previous 72 hours, use of vaginal products or any treatment with antibiotics in the previous 30 days were excluded from the study.

Following the local protocol, during specular exam women were checked for vaginal pH (range 4.0-7.0; Merck, Germany). Whiff test was performed by adding a 10% KOH solution to vaginal content in a clean cotton swab and results were expressed in positive, doubtful or negative. Vaginal smears from the mid-third lateral wall were confectioned. *Trichomonas vaginalis* infection was evaluated by wet mount microscopy immediately after sampling. Additional smear was transported to the reference laboratory where they were evaluated under phase-contrast microscopy for classification of the vaginal flora, according to previously described.[22] Briefly, lactobacillary grades (LBG) were classified as LBG I when *Lactobacillus* sp. morphotypes were predominant and considered as normal flora, LBG II in the presence of lactobacilli combined with significant amount of other bacterial species, and LBG III when the total replacement of the lactobacilli. Bacterial vaginosis was diagnosed on smears that presented the typical anaerobe granular microflora. Aerobic vaginitis was defined as the presence of small bacilli and/or cocci in pairs or chains, increased number of leukocytes, presence of toxic leukocytes and parabasal cells. Candidosis was diagnosed when *Candida* sp. morphotypes were observed on the smears.

Additionally to the local protocol for assessment of the vaginal flora, endocervical content was taken for detection of CTand NG by polymerase chain reaction (PCR). Samples were collected by rotating a cytobrush 360° three times and frozen at -20^{o}C in 1mL of a Tris-EDTA-Tween solution until analysis. DNA extraction was performed with previous digestion with

proteinase K and DNA integrity was confirmed by the amplification of beta-globin constitutive gene.[23] Infection by CT was detected by the amplification of a 201 bp sequence, using the primers CTP1 (5'-TAG TAA CTG CCA CTT CAT CA-3') and CTP2 (5'-TTC CCC TTG TAA TTC GTT GC-3')[24] and NG by a 390 bp sequence with the primers OH1 (5'-GCTACGCATACCCGCGTTGC-3') and OH3 (5'-CGAAGACCTTCGAGCAGACA-3').[25] All reactions were performed using de GoTaq Green Master Mix (Promega, Madison, WI), according to manufacturer's instructions. Positive controls were run simultaneously in all reactions and consisted of DNA CT extracted from culture of infected McCoy cells and standard strain of NG (ATCC® 19424). Amplicon size was determined by comparing to a standard size marker under UV transillumination.

For assessment of the cytokine levels, vaginal rinsing with 3mL of sterile saline solution was performed by allowing the contact of the liquid with the vaginal wall and recovering exactly 3mL with a sterile plastic pipette. Professionals were instructed how to perform this procedure and sample supernatants were obtained by centrifuging at 800 x *g* for 10 minutes and stored a -80°C until analysis. Measurement of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α was performed by enzyme-linked immunoabsorbent assay (ELISA) using Duo Set Kits (R&D Systems, Minneapolis, MN). Minimum detectable levels were 0.98 pg/mL, 3.27 pg/mL, 7.80 pg/mL and 6.43 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. Intra-assay variation coefficients were 7.4% for IL-1β, 9.8% for IL-6, 9.1% for IL-8 and 23.4% for TNF-α. Regarding inter-assay variability, respective coefficients for IL-1β, IL-6, IL-8 and TNF-αwere 5.3%, 4.1%, 5.1% and 25.5%.

Statistical analyses for comparison of cytokine levels between the groups with or without CT infection were performed with the non-parametric Mann-Whitney test. Significance level adopted was $p<0.05$. Tests were performed with GraphPad Prism version 5.00 (San Diego, California, USA). Post-hoc analysis showed a power test of 80%, considering the difference of IL-1β and IL-8 among the groups with normal flora and BV.

Results

Socio-demographic and behavioral characteristics and gynecological data from all women included in the study are shown in Table 1. The median age was 31 years, women mostly self-reported as white (68.1%), non-smokers (78.0%), sexually active by the time of enrollment (96.1%) and multiparous (63.2%).

We first compared the vaginal level of cytokine of women with BV and those with normal flora pattern in women non infected by CT. The levels of IL-1β, IL-6, IL-8 and TNF-α for both groups are shown on Table 2. Interleukin 1β had detectable levels in 65 (34.6%) and 164 (87.2%) samples from women with normal flora and BV, respectively. Interleukin-1β concentrations were significantly higher in women with BV (median 52.9pg/mL; range 0.0-1950.0) than normal flora (median 4.8pg/mL; range 0.0-373.1) (P<0.001). Levels of IL-6 were below the detection limit in most of normal (n=153, 81.4%) and BV samples (n=129, 68.6%). No difference in vaginal IL-6 levels was observed between BV (median 0.0 pg/mL; range 0.0-324.4) and normal flora (median 0.0 pg/mL; range 0.0-195.6). In relation to IL-8, most of the samples with normal flora (184, 97.9%) and BV (168, 89.4%) had detected levels. Comparison did not show differences among the groups with BV (median 234.8 pg/mL; range 0.0-2401.0.0) and normal flora (median 240.8 pg/mL; range 0.0-2010.0). Only few samples had detectable levels of TNF-α, 31 (16.5%) and 20 (10.6%) from normal and BV groups, respectively. Median TNF-α concentration did not differ between the groups (*P*>0.05).

According to the results shown in Table 2, there was no difference in cytokine levels between group of women with normal vaginal flora in relation to presence of CT infection (*P*>0.05). However, when analyzing women with microscopic finding of BV, there was a significant higher level of IL-1β (*P*=0.02), IL-6 (*P*=0.02) and IL-8 (*P*=0.03) in women with chlamydial infection in relation to women with only BV. No difference on TNF-α between these groups was identified (*P*=0.27).

Discussion

Many studies have shown that BV facilitates the acquisition of STIs.[10-14] However few works evaluated the inflammatory response through the measurement of the different pro-inflammatory profile cytokine in the vaginal content in the presence of STI alone and accompanied by BV.[19,20] Hence, our study focused in the interference of CT infection in the innate immune response to BV. A major effort was made in order to include only women with microscopic finding of exclusive BV, since presence of NG infection or other abnormalities in the vaginal flora, such as candidosis, intermediate flora and AV can interfere on local cytokine levels.[7,13,19]

The findings here presented regarding the vaginal levels of pro-inflammatory cytokine in BV in comparison with normal flora corroborate with previous studies that found increased IL-1β, but unchanged IL-6, IL-8 and TNF-α in BV. The mechanism interfering in the downstream of the inflammatory cascade after IL-1β release in VB has not been established. Previous reports suggested that microbial products such as sialidase could interfere in the IL-8 production in BV,[26] however this has not been confirmed by our previous study that showed that increased IL-8 can be detected in the presence of high sialidase levels.[6] It was recently demonstrated that other bacterial products, such as the short chain fatty acids (SCFAs) that are largely produced by anaerobes in bacterial vaginosis, can interfere with the TLR-mediated inflammatory response.[13]

Bacterial vaginosis was already recognized as an important risk factor for CT acquisition[10,11,13] as well as other STIs.[10-14] Additionally, it was already demonstrated that treatment of asymptomatic BV women reduces the incidence of CT when compared to women that suffered no intervention.[28] Considering that local increase of IL-1β levels is a known factor linked to increasing risk STIs acquisition[29] and that after BV treatment IL-1β level drops significantly[30,31] this can be considered one potential factor participating of the mechanisms

facilitating CT acquisition in BV. Other factor that can predispose for STIs acquisition is the degradation of the cervical mucus by sialidases and mucinases found in high concentration in BV, by diminishing the viscosity of the vaginal content and facilitating microrganisms invasion.[32]

In this study, we could also demonstrate BV in the presence of CT infection has a diverse cytokine profile, as it is accompanied by increases in IL-6 and IL-8 levels. Our data is in disagreement with previous finding that CT does not increase vaginal IL-8 in BV.[19] However this study was performed with a smaller sample size than ours what make these studies incomparable. Although the immune regulation mechanisms in BV are not yet characterized, it does not appear to be sufficient to block the cytokine cascade during CT infection. Also, we can consider that a higher endocervical production of pro-inflammatory cytokines triggered by CT infection may stimulate vaginal production of IL-6 and IL-8 despite the immune regulation in response to BV. In fact, several studies showed that vaginal fluid from women with BV are more stimulatory in activating the cytokine cascade *in vitro* when compared to normal flora contents.[33,34] Our finding regarding unchanged levels of pro-inflammatory cytokines in response to CT in the presence of normal flora reinforces the importance of lactobacilli protection against STIs acquisition, as demonstrated by previous studies through the significant lower rates of STIs in lactobacilli colonized vaginal flora.[35,36]

Based on these findings, we conclude that the production of IL-1β, IL-6 and IL-8 in BV are modulated by the presence of chlamydial infection. In addition, normal flora has important mechanisms for the maintenance of a balanced vaginal environment even in the presence of CT.

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Table 1. Socio-demographic and behavioral characteristics and gynecological data from 512 women included in the study.

Variable	Median (range) or N/Total evaluated* (%)
Age (years)	31 (18-51)
White	290/426 (68.1)
Married	344/487 (70.6)
Education level (years at school)	10 (0-18)
Paid activity	289/479 (60.3)
Smoking habit	108/490 (22.0)
Sexually active	467/486 (96.1)
Multiparous	307/486 (63.2)

*Total of women may vary due to refusal to answer or answer given was other than the categories included on the table.

Table 2. Pro-inflammatory cytokine levels in the vaginal samples from women with normal flora and bacterial vaginosis according to the status of *Chlamydia trachomatis* infection.

Cytokines (pg/mL)	**NF (n=188)**	**NF with CT (n=68)**	***P****	**BV (n=188)**	**BV with CT (n=68)**	***P****
IL-1β	4.8 (0.0-373.1)	11.1 (0.0-396.8)	0.21	52.9 (0.0-1950.0)	92.3 (0.0-1536.0)	*0.02*
IL-6	0.0 (0.0-195.6)	0.0 (0.0-38.3)	0.81	0.0 (0.0-324.4)	0.0 (0.0-220.9)	*0.02*
IL-8	240.8 (0.0-2010.0)	372.4 (0.0-1466.0)	0.11	234.8 (0.0-2401.0)	300.9 (0.0-2000.0)	*0.03*
TNF-α	0.0 (0.0-142.2)	0.0 (0.0-6.4)	0.14	0.0 (0.0-45.6)	0.0 (0.0-114.7)	0.27

NF: Normal flora;
BV: Bacterial vaginosis;
CT: *Chlamydia trachomatis*;
*Mann-Whitney test; P<0.05 considered as significant.

7. Conclusões

Considerando os resultados obtidos neste estudo a partir do tamanho amostral incluído e das metodologias empregadas, pode-se concluir que:

- A maior participação das espécies *A. vaginae*, *Megasphaera* sp. e *Leptotrichia* sp. na microbiota vaginal não está associada a manutenção do ambiente vaginal equilibrado, já que diferenças em suas concentrações relativas não modulam parâmetros relacionados a imunidade inata, atividade de sialidase e quantidade bacteriana;

- A infecção clamidiana modula a produção de citocinas pró-inflamatórias na vaginose bacteriana, mas não na microbiota vaginal normal, demonstrando, portanto, que a microbiota normal apresenta importantes mecanismos para a manutenção do ambiente vaginal equilibrado, apesar da infecção por *Chlamydia trachomatis*.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convidamos a senhora para participar da pesquisa "**Aspectos clínicos, microbiológicos e da imunidade inata dos casos de vaginose bacteriana com predomínio de bactérias produtoras de ácido lático"**,que tem por objetivo analisar alguns aspectos relacionados à infecção genital denominada Vaginose Bacteriana e suas variações presentes nos diferentes casos das mulheres atendidas suas variações presentes nos diferentes casos das mulheres atendidas nos ambulatórios de Ginecologia do CAISM – UNICAMP e do HC da Faculdade de Medicina de Botucatu - UNESP. Sua participação implicará em responder as questões da entrevista, e submeter-se a exame ginecológico, que serão realizados pela clínicos durante a consulta. Para o exame ginecológico, será necessária a introdução de um aparelho de metal, estéril, conhecido como "bico de pato" (espéculo), que afastará as paredes vaginais, a fim de permitir a visualização das mesmas e do colo do útero, bem como a coleta de amostras (secreção) para exames laboratoriais. O material da parede vaginal e do colo do útero será coletado por meio de um cotonete, para verificação dos tipos de bactérias presentes. Será, também, realizada coleta de material cérvico vaginal através da lavagem do local com solução fisiológica. Esta pesquisa é de responsabilidade da doutoranda Camila Marconi, sob orientação da Profª Drª. Márcia Guimarães da Silva, do Departamento de Patologia da Faculdade de Medicina de Botucatu, UNESP e com colaboração da Profa. Adjunta Anaglória Pontes e da Profa. Ana Gabriela Pontes de Souza do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Botucatu, UNESP e do Prof. Titular Paulo Cesar Giraldo, do CAISM, UNICAMP.

Pelo presente instrumento, eu _____ devidamente esclarecida, ciente dos procedimentos aos quais serei submetida, não restando quaisquer dúvidas a respeito do lido e explicado, e ciente, também, de que as informações serão utilizadas exclusivamente pelas pesquisadoras, que manterão sigilo sobre minha identidade, e que as mesmas estarão disponíveis para responder a quaisquer perguntas e de que **posso retirar este consentimento a qualquer hora sem prejuízo do meu atendimento neste Hospital,** firmo meu **CONSENTIMENTO LIVRE E ESCLARECIDO,** concordando em participar da pesquisa proposta.

Esse documento, após aprovação do CEP, será elaborado em duas vias, sendo uma entregue a paciente pesquisada e outra será mantida em arquivo pela pesquisadora.

Botucatu,_____ de _____de 20__

_____

Assinatura da paciente

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Profª. Drª. Márcia G. da Silva
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Jd. Chácara dos Pinheiros,Botucatu,SP
Fone: 14 3814-2417
e-mail: mgsilva@fmb.unesp.br

Questionário de coleta

Nome.....

RG.....

Idade ....

Etnia: ☐ Branca ☐ Não-branca

Estado civil: ☐ União-estável ☐ Solteira

Escolaridade (anos na escola).....

Profissão..... ....

Fumante ☐ Sim ☐ Não Cigarros/dia (no ano anterior):.....

Antecedentes ginecológicos:

Número de parceiros sexuais (1 ano) ☐ 1 ☐ 2 ☐> 2

Freqüência de relações sexuais.....

História de VB anterior.....

História de DST anterior.....

Métodos contraceptivos (4 meses anteriores):

☐ DIU ☐ Pílula/Tipo..... ☐ Outro .....

Antecedentes obstétricos:

Paridade: ☐ Nulípara ☐ Multípara

Intercorrências em gestações anteriores (parto prematuro, rotura prematura de membranas, aborto espontâneo, morte fetal): .....

Responsável pela coleta

unesp Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18 618-970
Fone/Fax: (0xx14) 3811-6143
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e-mail coordenadoria: tsarden@fmb.unesp.br

Registrado no Ministério da Saúde em 30 de abril de 1997

Botucatu, 11 de fevereiro de 2009. Of. 36/09-CEP

Ilustríssima Senhora
Profª Drª Márcia Guimarães da Silva
Departamento de Patologia da
Faculdade de Medicina de Botucatu

Prezada Drª Márcia,

Informo que o Projeto de Pesquisa "**Aspectos clínicos, microbiológicos e da regulação da umunidade inata na vaginose bacteriana**", a ser conduzido por Camila Marconi, orientada por Vossa Senhoria, com a colaboração do Prof. Dr. Paulo César Giraldo, recebeu do relator **parecer favorável com condição**, aprovado em reunião de 06/10/2008.

Condição: Projeto de Pesquisa aprovado em reunião do CEP de 06/10/2008, condicionado seu início após apreciação e aprovação pelo CEP da UNICAMP, conforme orientação contida no ofício 2440-CONEP/CNS/MS de 24 de setenbro de 2.008.

Situação do Projeto: **APROVADO COM CONDIÇÃO. Apresentar Relatório Final de Atividades ao final da execução deste projeto.**

Atenciosamente,

Prof. Dr. Trajano Sardenberg
Coordenador do CEP.

CEP, 16/04/09.
(Grupo III)

PARECER CEP: Nº 282/2009 (Este nº deve ser citado nas correspondências referente a este projeto)
CAAE: 0001.0.357.146-09

I - IDENTIFICAÇÃO:

PROJETO: **"ASPECTOS CLÍNICOS, MICROBIOLÓGICOS E DA IMUNIDADE INATA NA VAGINOSE BACTERIANA".**
PESQUISADOR RESPONSÁVEL: Camila Marconi
INSTITUIÇÃO: CAISM/UNICAMP
APRESENTAÇÃO AO CEP: 13/04/2009
APRESENTAR RELATÓRIO EM: 28/04/10 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Ampliar o conhecimento sobre a microbiologia envolvida nos casos e vaginose baceteriana e sua relação com a modulação da resposta imune inata e com a atividade enzimática bacteriana.

III - SUMÁRIO

Serão incluídas nesse estudo 628 pacientes atendidos no Ambulatórios de Infecções Genitais do Setor de Ginecologia do CAISM/UNICAMP, e dos Ambulatórios de Ginecologia do Hospital das Clínicas da Faculdade de Medicina de Botucatu, UNESP. A detecção e quantificação relativa das espécies Atopobium vaginae, Leptotrichia, Megasphaera sp. serão realizadas pela técnica de PCR em tempo real. Alem disso, esses microrganismos serão semi-quantificados nos esfregaços vaginais através da técnica de FISH. Para avaliação da resposta imune local serão quantificados os níveis de citocinas inflamatórias, Il1b, IL-6, IL-8 e TNF-a, presentes no lavado cérvico-vaginal por ELISA, além da avaliação de polimorfismo no gene da IL1b. As atividades de sialidades e prolidases serão quantificadas através de método enzimáticos específicos. Analise dos dados: a comparação entre níveis de citocinas e de atividades de sialidases e prolidases nos grupos de VB será realizada através do teste não paramétrico de Mann-Whitney. A comparação das freqüências de polimorfismos nas pacientes dos grupos com maior ou menor produção de citocinas será realizada pelo teste de Qui-quadrado. As análises estatísticas serão realizadas respeitando os pressupostos determinados pelos resultados obtidos.

IV - COMENTÁRIOS DOS RELATORES

Trata-se de um projeto para tese de doutorado, cujo objetivo é ampliar o conhecimento sobre a microbiota vaginal em mulheres com VB. O protocolo esta bem elaborado, será desenvolvido em dois centros universitários, foi apreciado pelo CEP/Unesp e aprovado com a condicional de aprovação neste CEP para início da pesquisa. Conta com orçamento parcial já aprovado pela FAPESP. O Termo de Consentimento Livre e Esclarecido está redigido em

linguagem acessível e contempla os aspectos necessários, não levando riscos às mulheres participantes.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, bem como ter aprovado o Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Aprovado "ad referendum" em 16 de abril de 2009.
A ser homologado na IV Reunião Ordinária do CEP/FCM, em 28 de abril de 2009.

Profa. Dra. Carmen Silvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

Comitê de Ética em Pesquisa - UNICAMP
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