Association of sexually transmitted infections, Candida species, gram-positive flora and perianal flora with bacterial vaginosis

Running title: Bacterial vaginosis and associated factors

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SUMMARY

Bacterial vaginosis (BV) is characterised by depletion of the normal *Lactobacillus* spp. and overgrowth of commensal anaerobic bacteria. We investigated the composition of vaginal microbiota and their association with BV in women of reproductive age. Vaginal samples from 1197 women were analysed, whereby n=451 patients had normal flora and n=614 were diagnosed with BV, the remaining patients were diagnosed with having either intermediate flora (n=42) or dysbacteriosis (n=90). The reported results show that pathogens are associated with BV. This knowledge will further expand our understanding of events leading to BV, which may lead to more effective prevention and treatment strategies.

*KEY WORDS:* Bacterial vaginosis, Epidemiology, Nucleic acid amplification tests, Sexually transmitted infections, Perianal flora
Introduction

Bacterial vaginosis (BV), a disorder that causes infection or inflammation of the vagina accompanied by such symptoms as itching, burning, and vaginal discharge, is the most common vaginal syndrome affecting fertile, premenopausal and pregnant women (Mastromarino et al., 2013; Lamont et al., 2012). It is also important to know that the majority of women with BV do not have symptoms. If left untreated, BV can lead to other complications, including pelvic inflammatory disease (Sharma et al., 2014), infertility, preterm birth and low birth weight (Sirot et al., 2014). BV is characterised by depletion of the normal Lactobacillus spp. and overgrowth of commensal anaerobic bacteria (Marcone et al., 2008). Thus, vaginitis is considered to be a warning symptom indicating the onset of such complications and should be treated promptly.

Nucleic acid amplification tests (NAATs) have proven superior to conventional clinical approaches - using the Amsel criteria and/or the Nugent gram stain scoring system (Sha et al., 2005) - in the diagnosis of vaginal smears, enabling diagnostics with enhanced sensitivity and specificity. It is generally accepted that healthy vaginal flora is dominated by Lactobacillus spp., however, an overgrowth of (facultative) anaerobic bacteria, such as Gardnerella vaginalis and Atopobium vaginae, represent the transition from normal vaginal flora to BV (De Backer et al., 2010; Dacu et al., 2013) The order of events leading to the development of BV remains poorly characterised and it is unclear whether compositional changes in Lactobacilli spp. and anaerobe bacteria are the only contributing factors to BV. Our goal was to investigate whether any association exists between BV and other infectious pathogens such as sexually transmitted infection (STI), Candida spp., gram-positive flora and perianal flora (gut flora).

MATERIAL AND METHODS

Study design

This was a cross-sectional study designed to determine the aetiology of vaginal symptoms among women in Amsterdam, the Netherlands.

Patients
A total of 1197 female patients of reproductive age (≤ 50) with clinical suspicion of BV and STI were tested between September and December 2013. The vaginal specimens were self-collected swabs as a pair in a lysis buffer eNAT and transport buffer Eswab (Copan, Brescia, Italy) for Real Time PCR (RT-PCR) / multiplex PCR (m-PCR) and cultures, respectively (Menard et al., 2012). The self-collecting swabs were given to patients by their GPs accompanied with a “Laboratory & Instruction” form. In short, GPs would fill out the form and give a quick instruction such as using the vaginal swab for sampling in the morning before using the bathroom, snapping the swab followed by inserting the swab in the buffer container to elute.

**Real time and multiplex-PCR**

The bacterial vaginosis targets were based on quantitation of DNA of *Gardnerella vaginalis* (GV), *Atopobium vaginae* (AV), *Lactobacillus* spp. (LS), and the total amount of bacteria (TB) in clinical materials. The categorization of the findings based upon RT-PCR are as follows: 1. Normal Flora (NF), normal distribution of vaginal flora, whereby the total amount of bacteria is equal to the detected amount of the *Lactobacillus* spp. (Ct ± 2). 2. Intermediate Flora (IF), shifted distribution within the vaginal flora whereby the *Lactobacilli* spp. are decreased and the amount of detected *Gardnerella vaginalis* and / or *Atopobium vaginae* is increased without any significant changes. 3. Bacterial Vaginosis (BV), shifted distribution within the vaginal flora whereby the *Lactobacilli* spp. are decreased and the amount of detected *Gardnerella vaginalis* and / or *Atopobium vaginae* is significantly increased. 4. Dysbacteriosis (Dys) whereby the total amount of detected *Lactobacilli* spp. is significantly decreased compared to the total amount of bacteria, but *Atopobium vaginae* and / or *Gardnerella vaginalis* play no significant role. For the BV and STI, m-PCR for AmpliSens Florocenosis/Bacterial vaginosis-FRT PCR (RT-PCR) and *T.vaginalis*/{*N.gonorrhoeae/C.trachomatis* (TV/NG/CT) MULTIPRIME-FRT PCR (m-PCR) kits (AmpliSens biotechnologies, Moscow, Russia) were used and performed on Roche Aurora Flow according to the manufacturer’s specifications (Vahidnia et al., 2014)

**Selective culture and media**

For culture diverse selective and non-selective plates were used, such as: colistin-aztreonam agar plate for gram-positive microorganisms; a chromogenic Brilliance *Candida* agar and a chocolate agar plate (non-selective) (Oxoid B.V., Badhoevedorp, the Netherlands) for gram-negative bacteria; a selective gonococcal agar plate (BD diagnostics, Erembodegem,
Belgium). These plates were inoculated on the same day and subsequently incubated at 36°C in a 5% CO₂-enriched environment. Growth was observed after 24 hours of incubation, with negative growth after 48 hours. Bacteria were identified according to standard operating procedures (Garcia and Isenberg, 2007); susceptibility testing was performed on the BD Phoenix system (BD Diagnostics, Erembodegem, Belgium) and EUCAST guidelines were applied when available (http://www.eucast.org/).

The human vagina is lined with several layers of epithelium cells. Many indigenous microorganisms colonize these surfaces. Accurate diagnosis of genital infections from the female genitalia depends on the separation of microbial pathogens from the normal genital microbiota. Many adult female genital tract infections arise from endogenous microorganisms, the pathogenicity of which has been activated by host factors or other microorganisms (Garcia and Isenberg, 2007). Culture-based analyses have contributed to the knowledge on the microbes inhabiting the vagina and the understanding of infectious diseases that affect the genital tract. However, culture techniques are challenging due to the fact that the perianal (predominant growth of (anaerobic) enteric gram-negative rods) and the vaginal flora (predominant growth of *Lactobacilli*) are both poly-microbial. Identification and antibiotic susceptibility of the cultured bacteria were identified by their relative abundance in the vaginal sample taken.

Statistical analyses

Statistical analyses were performed using the IBM SPSS statistical software (version 22). Binary logistic regression analyses based on values of other pathogens were used to predict the event leading to BV. p values <0.05 were considered significant.

**RESULTS**

The study population was divided into four groups based on RT-PCR results for NF, IF, BV and Dys. The group with NF results (n=451) had an average age of 31 (range: 16-50) and a median age of 30 years. The group that tested positive for IF (n=42) had an average age of 29 (range: 16-44) and a median age of 28 years. The group that tested positive for BV (n=614) had an average age of 30 (range: 15-50) and a median age of 29 years. The group diagnosed with dysbacteriosis had an average age of 33 (range: 15-50) and a median age of 32 years. For the NF group a total of 15 patients tested positive for CT or NG, while the BV group showed a total of 89 patients tested positive for either CT, NG or TV (m-PCR) (Table 1).
The various culture results of vaginal swabs for gram-positive flora and perianal flora were pulled based on the aetiology of detected microorganisms. The gram-positive flora consisted of *haemolytic streptococcus A, B and G, staphylococcus aureus* and *enterococci*. The perianal flora consisted of *E-coli, Pseudomonas aeruginosa, Citrobacter pneumonia, Klebsiella pneumonia* and faecal flora. Faecal flora (or normal enteric flora) consist for 99% out of anaerobe microorganisms. These organisms (*Bacteroides, Clostridium, peptostreptococci, peptococci* and others) far exceed the number of aerobes. The role of normal flora is extremely important as a host defence resisting colonization by potentially pathogenic invaders.

Of the total tested *Candida* spp., for NF 93.5% were *Candida albicans* and the remaining 6.5% were diagnosed as non-*albicans*. In the BV group, 96.7% of the tested *Candida* belonged to *albicans* and the remaining 3.3% were non-*albicans*.

When analysing the various pathogen groups in a binary logistic regression model and comparing IF, BV and Dys to NF, presence of STI and perianal flora remained associated to BV, with p-values of <0.001 and <0.05, respectively, and an overall odds ratio of 1.4 for all the variables in the equation. Prevalence of individual STI pathogens in the BV-group, namely CT and TV, was significant, with p-values of <0.001 and p<0.05 for NG. Furthermore, presence of *Candida* spp. and gram-positive flora had no significant correlation to BV, while their correlation to IF and Dys were found to be significant (Table 2).

**DISCUSSION**

Various studies have shown the diagnostic value of NAATs in predicting and diagnosing BV, whereby the prevalence of vaginal pathogenic bacteria is accompanied by a depletion of *Lactobacillus spp.* (Ling et al., 2013). Our findings are consistent with earlier studies investigating the correlation between BV and STI, especially TV (Gallo et al., 2012; Fichorova et al., 2013). In our test panels, TV was completely absent in the control group NF. However, our findings suggest that the presence of perianal flora and STI indicate that there is a correlation between the presence of perianal flora, STI with BV, whereas *Candida* and gram-positive flora have no significant effect. This finding is partly supported by other studies postulating that the mechanism behind CT persistence in humans may be a result of ongoing CT infection in the gut and that re-infection of the genital tract is a result of auto-inoculation.
from the lower GI tract (Rank and Yeruva., 2014). In such cases, treatment policies should be revised to a course of doxycycline rather than azithromycin. Given that BV is not limited to a single causative infectious agent (Lamant et al., 2011) and most BV-associated bacteria may be found in women without disease, the utility of standard culture for diagnosis is limited; the positive predictive value of a positive G. vaginalis culture is less than 50%, whereas neither the so-called Amsel’s criteria nor the Nugent scoring system is discriminative enough (Hillier 1993). The use of cultivation-independent techniques has shown that women with BV exhibited considerably greater bacterial diversity than women without. The exact mechanism behind the association between BV and the adverse effects is still unclear; whether BV-associated bacteria are capable of ascending into the intrauterine cavity is an issue that requires further study.

ACKNOWLEDGEMENT

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REFERENCES


Table 1. Number of positive samples for STI, *Candida spp.*, gram positive flora and perianal flora detected by either RT/m-PCR or diverse (selective) culture in clinical specimens.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Number of positive samples</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>STI</td>
<td><em>Candida</em></td>
<td>Gram pos flora</td>
<td>Perianal flora</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>CT</td>
<td>NG</td>
<td>TV</td>
<td>Spp.</td>
<td>flra</td>
<td>flra</td>
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<tr>
<td>Normal Flora (NF)</td>
<td>451</td>
<td></td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>147</td>
<td>33</td>
<td>40</td>
<td></td>
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<tr>
<td>Prevalence (%)</td>
<td>37.68</td>
<td></td>
<td>3.33</td>
<td>32.59</td>
<td>7.32</td>
<td>8.87</td>
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<tr>
<td>Intermediate Flora (IF)</td>
<td>42</td>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>20</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>3.51</td>
<td></td>
<td>9.52</td>
<td>47.62</td>
<td>16.67</td>
<td>7.14</td>
<td></td>
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<td>Bacterial vaginosis (BV)</td>
<td>614</td>
<td></td>
<td>48</td>
<td>12</td>
<td>29</td>
<td>174</td>
<td>62</td>
<td>84</td>
<td></td>
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<tr>
<td>Prevalence (%)</td>
<td>51.29</td>
<td></td>
<td>14.5</td>
<td>28.34</td>
<td>10.1</td>
<td>13.68</td>
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<tr>
<td>Dysbacteriosis (Dys)</td>
<td>90</td>
<td></td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>12</td>
<td>20</td>
<td>13</td>
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<tr>
<td>Prevalence (%)</td>
<td>7.52</td>
<td></td>
<td>5.56</td>
<td>13.33</td>
<td>22.22</td>
<td>14.44</td>
<td></td>
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<tr>
<td>Total</td>
<td>1197</td>
<td></td>
<td>65</td>
<td>16</td>
<td>32</td>
<td>353</td>
<td>122</td>
<td>140</td>
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</tr>
</tbody>
</table>
Table 2. Correlation between occurrence of IF, BV and Dys and the contributing factors by other pathogens.

<table>
<thead>
<tr>
<th>STI</th>
<th>Candida</th>
<th>Gram pos flora</th>
<th>Perianal flora</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BV</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Dys</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

IF, intermediate flora; BV, bacterial vaginosis; Dys, dysbacteriosis.