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The Role of Bacterial Vaginosis and Trichomonas in HIV Transmission Across The Female Genital Tract

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Abstract

Bacterial vaginosis (BV) and *Trichomonas vaginalis* (TV) infection are both very common and are associated with increased risk of sexual transmission of HIV. There are several mechanisms by which BV and TV could affect susceptibility including inducing pro-inflammatory cytokines and disrupting mucosal barrier function. This review highlights recent advances in our understanding of how these genital conditions lead to an increased risk of HIV infection in women.

Keywords

Bacterial vaginosis; HIV; Inflammation; *Trichomonas vaginalis*

Bacterial Vaginosis (BV) and HIV

The lower genital tract microbiota is a dynamic bacterial community that is influenced by many factors including age, sexual activity, hormones, infection, the immune system and individual hygiene practices. The microbiota of many women is composed predominantly of lactobacilli, which are believed to contribute to the health of the vagina by producing lactic acid and by out-competing pathogenic organisms. Lactobacilli can also produce H₂O₂, which is also thought to promote vaginal health [1-3]. Bacterial vaginosis (BV), in contrast, is a condition in which the types and the relative proportions of bacteria are altered in the lower genital tract. BV is common with 8-23% of women of reproductive age affected. Interestingly, more than half of BV+ women report no symptoms [4].

The bacterial communities comprising BV can vary substantially from individual to individual. However, studies using conventional culture methods, as well molecular techniques (i.e. direct sequencing or PCR of the 16S rRNA genes) show that the microbiota in BV mostly consist of bacteria in the order *Clostridiales*, *Gardnerella vaginalis*, *Mycoplasma hominis*, *Prevotella sp.*, *Atopobium*, and *Mobiluncus sp.* ([3, 5, 6] and Fig. 1). Antibiotics such as metronidazole and clindamycin are used to treat BV, but unfortunately, as many as 30% of women experience relapse within the first 3 months after treatment [4].

BV can be identified by a variety of methods [3]. Clinically, the Amsel test is commonly used where four criteria are assessed: vaginal fluid pH >4.5, presence of clue cells (bacteria-coated epithelial cells), homogenous thin discharge, and a whiff test that yields an amine odor when KOH is added to vaginal fluid [7]. If three of these tests are positive, BV is

diagnosed. BV can also be determined by the Nugent scoring system, where a score is applied to Gram stains of vaginal smears to visually estimate the numbers of lactobacilli and BV-associated bacteria. According to this system, a Nugent score of 0-3 is considered healthy, a score of 4-6 is intermediate and a score of 7-10 signifies BV [8].

In most women, a healthy microbiota consists predominantly of lactobacilli such as *L. crispatus*, *L. jensenii*, *L. gasseri* or *L. iners*, although several recent cross-sectional studies have shown that *Lactobacillus* sp. are not the predominant bacteria in a significant number of “healthy” women [3, 9-12]. A recent study sequenced the genital microbiota of 396 asymptomatic North American women and found there were generally five types of bacterial communities, dominated by either *L. iners*, *L. crispatus*, *L. gasseri*, or *L. jensenii*, as well as a fifth group that was not dominated by lactobacilli but instead consisted of mostly anaerobes and *G. vaginalis* [12]. In a deep sequencing analysis of the microbiota of a group of HIV+ African women, *L. crispatus* and *L. iners* were found to strongly associate with a normal vaginal microbiota [13].

Studies Showing an Association between BV and HIV Infection

BV is associated with the acquisition and transmission of the human immunodeficiency virus (HIV), as well as other sexually transmitted pathogens [4, 14-19]. Given BV's prevalence, especially in areas most afflicted by heterosexual transmission of HIV [20], BV is recognized as one of the most important factors affecting HIV susceptibility in women.

The association between BV and the risk of HIV infection in women has been demonstrated in several cross-sectional [21-23] and longitudinal studies [24, 25] where HIV was more frequent in BV+ women than those without BV. Moreover, sub-Saharan African women, whose vaginal microbiota was not dominated by lactobacilli, were found to be 2-3 times more likely to be infected with HIV, even when other HIV risk factors were taken into account [20, 24-26]. A 2008 meta-analysis of twenty three studies found that BV increased the risk of HIV infection in women by 60% [15]. Several groups have also demonstrated that among HIV sero-positive women, those with BV or with low levels of lactobacilli shed more HIV viral particles in their vaginal secretions [27-29]. Moreover, studies show that vaginal fluids collected from BV+ women can stimulate HIV expression *in vitro* [30].

Interestingly, within the bacterial communities that can comprise BV, there may be some that are even more strongly associated with increased levels of HIV expression in genital fluids than others. In our own studies, we found there was a significant positive correlation between levels of *M. hominis* and genital HIV shedding in a multivariate analysis controlling for plasma viral loads [29]. In fact, our data showed that women with low lactobacilli and high *M. hominis* had 100-fold higher levels of genital HIV than those with high lactobacilli and low *M. hominis*. However, we found no significant relationship between *G. vaginalis* and genital tract HIV. These findings suggest that BV in general, and certain types of BV-associated bacteria in particular, can have a profound effect on HIV expression in the genital tract. This is important because the level of expression of HIV in the genital fluid is a critical factor in female to male transmission of HIV.

The studies above clearly establish a link between BV and rates of HIV infection and expression. However, the mechanisms by which BV contributes to increased susceptibility and shedding remain to be determined. An animal model could be helpful in elucidating the relationship between HIV and BV. The vaginal infection of rhesus and pigtailed macaques with the simian immunodeficiency virus (SIV) or the simian-human immunodeficiency virus (SHIV) is used extensively as a model to investigate HIV sexual transmission. Recent studies have addressed the factors that affect macaques' lower genital tract microbiota and their metabolic products. Those studies show that in macaques, similar to women with BV,

the lower genital tract is not dominated by lactobacilli but is polymicrobial ([31-33] and Fig. 2). In addition, like BV+ women, macaques' vaginal fluids have low levels of lactic acid and glycogen [34], and a relatively high pH [31, 32, 35]. Recently, Lagenaur *et al.* showed that introduction of *L. jensenii* expressing cyanovirin-N (an anti-HIV protein) into the macaques lower genital tract significantly lowered the infection rate of macaques vaginally challenged with SHIV [36], although it remains to be determined how much of this protection is due solely to colonization by lactobacilli.

Interestingly, in our analysis of the macaques' lower genital tract microbiota by direct sequencing, we also found some differences and similarities in the types and the frequencies of bacteria colonizing the lower genital tract of rhesus and pigtailed macaques, and some suggestion that the microbiota could be dependent on the facility in which they were housed (Fig. 2). Such variations may affect susceptibility to retrovirus infection and indicate that further studies are needed.

Possible Mechanisms of BV'S Effects on HIV

In this section, we highlight some recent studies that enhance our understanding of the mechanisms by which BV might affect HIV acquisition and transmission.

It is widely believed that the acidic environment of the vagina in women afforded by lactobacilli contributes to the protection against HIV infection [37, 38]. Indeed, while a healthy vagina is characterized as having a pH of less than 4.5, BV is marked by its relatively higher pH (pH > 4.5). It has also been suggested that a decrease in H₂O₂-producing lactobacilli in BV might contribute to increased rates of HIV infection in BV+ women [14, 39, 40]. However, this view is challenged by recent studies unable to detect H₂O₂ production by lactobacilli under the hypoxic conditions of the vagina, calling into question the relevance of H₂O₂ *in vivo* [41, 42].

The multimicrobial nature of BV is also believed to induce a pro-inflammatory environment, consisting of cytokines and toll-like receptor (TLR) ligands, that is conducive to HIV propagation ([43] and Fig. 3). Several groups have reported that the levels of pro-inflammatory cytokines are elevated in BV+ vaginal fluids (reviewed in [43]). Many of these studies show an increase in IL-1 levels in the vaginal fluids collected from BV+ women, while others also report an increase in IL-8 and IL-1 levels though the latter cytokines are not consistently observed to be elevated [44-49]. BV+ vaginal fluids also tend to have more RANTES and IL-6, although in most cases the level of these inflammatory mediators fails to reach statistical significance when compared to samples collected from BV-negative women [43]. In addition, genital secretions from women with BV can stimulate cells through TLR2 [50], suggesting that gram positive bacterial products such as peptidoglycan may be involved in inducing the proinflammatory cytokines in BV.

It has been hypothesized that pro-inflammatory cytokines may increase susceptibility to HIV infection by causing damage to the vaginal mucosa, thereby allowing HIV to breach the epithelial barrier [17, 20]. Once HIV gains entry to the underlying tissue, it likely infects CD4+ lymphocytes, macrophages, and dendritic cells, which are recruited to the site of inflammation [20]. Recently, Ballweber *et al.* showed that Langerhans cells in the vaginal epithelium could pick up infectious HIV and shuttle it to CD4+ T cells without the Langerhans cells becoming infected [51]. In addition, both TLR ligands and pro-inflammatory cytokines can activate NF- κ B, which has been shown to increase HIV expression in cells [52, 53]. These data support the hypothesis that BV provides an inflammatory environment that can promote HIV infection, and increase viral expression. It has therefore been suggested that inhibiting inflammatory innate immune responses might be important in preventing HIV dissemination [54].

BV-associated anaerobes have also been shown to produce large quantities of short chain fatty acids (SCFAs), including acetic and butyric acids. These small compounds are found at millimolar concentrations in the vaginal fluids of BV+ women (reviewed in [55], and can cross the cell membrane by non-ionic diffusion [56]. SCFAs, particularly butyric acid, may also be transported into cells by a class of transporters known as monocarboxylate transporters or MCTs, that have a wide tissue distribution that includes epithelial cells and leukocytes [57-62]. GPR43, a member of the G-protein-coupled receptor family that is expressed on the surface of monocytes and neutrophils, has also been shown to bind SCFAs and induce signaling [63-65].

SCFAs can regulate a myriad of immune responses, including cell migration, cytokine production, phagocytosis, and apoptosis in a type- and concentration-dependent manner (reviewed in [55]). Using concentrations of these compounds that are present in BV+ vaginal fluids, we found that high levels of SCFAs (20 mM) induce the production of IL-8, IL-6 and IL-1 by peripheral blood mononuclear cells (PBMCs) *in vitro* (Fig. 4 and [66]). In addition, we found that even at low levels, SCFAs enhanced the production of IL-8 and TNF α when combined with TLR2 and TLR7 ligands, but not TLR4 ligands [66]. Because of their far-reaching effects on immune responses and gene expression [67-72], it will be important to learn how SCFAs might affect HIV infection and expression in target cells. Indeed we found that butyric acid decreased both the formation of HIV provirus and the production of HIV viral protein p24 by macrophages (Fig. 5). These findings suggest that SCFAs may play opposing roles in HIV susceptibility: On the one hand they may induce a pro-inflammatory milieu in the vaginal mucosa, and thereby increase the rate of HIV infection. On the other hand, SCFAs may directly reduce the infection of target cells. It remains to be determined why different SCFAs have such a wide array of effects on immune cell responses.

In addition to inducing pro-inflammatory cytokines, BV-associated microbiota produce a number of enzymes and byproducts such as sialidases, glycosidases, and proteases [14]. These microbial compounds can compromise the host immunity to pathogens by 1) breaking down mucin, a component of the protective mucous lining the genital tract, 2) by degrading innate anti-microbial factors present in mucous such as secretory leukoprotease inhibitor (SLPI), α -defensins, and soluble IgA, or 3) by inactivating immune cells [73, 74]. Additionally, these enzymatic activities, which facilitate microbial attachment to the mucosal surfaces and provide nutrition for the bacteria, may disrupt the integrity of the vaginal epithelium. This, in turn, can allow HIV to gain entry into the underlying parenchyma and increase susceptibility to HIV infection. Interestingly, the presence of some pro-inflammatory cytokines, such as IL-1 and IL-8, has also been associated with an increase in the levels of BV-associated hydrolytic enzymes [75].

Trichomonas Vaginalis (TV) and HIV

Trichomonas vaginalis (TV) is the most common non-viral sexually transmitted infection with the World Health Organization estimating 173 million annual cases in 1999 [76]. A study of US women between the ages of 14 and 49 from 2001 to 2004 estimated the prevalence of trichomoniasis at 3.2% [77]. Strikingly, over 80% of cases were asymptomatic at the time of testing [77]. TV infection has been previously regarded by some as a mere nuisance, possibly in part due to the fact that it can be asymptomatic in so many. But more recent awareness that TV is associated with increased susceptibility to HIV infection and other severe problems such as preterm birth, as well as the frequency of TV infection, have led to the realization that TV needs a stronger public health response [76]. The immunology, epidemiology and biology of TV infection have been reviewed recently [78, 79].

Studies Showing an Association between TV and HIV Infection

A number of studies have shown an increase in HIV sero-conversion associated with TV infection. In a longitudinal study, Laga *et al.* [80] found increased HIV sero-conversion was associated with TV in 531 HIV-1 negative female sex workers in Kinshasa, Zaire that were screened monthly. A prospective study of 1,335 Kenyan women that were monitored monthly for a median of 566 days showed in multivariate analysis that infection with TV was associated with a 1.52 fold increased risk of HIV-1 seroconversion [81]. In a case control study, Van Der Pol *et al.* [82] used PCR to detect TV in a general population cohort of women from Uganda and Zimbabwe. Multivariate analysis showed an adjusted odds ratio for HIV acquisition of 2.74. Mavedzenge *et al.* showed that TV increased the risk of HIV sero-conversion in women from South Africa and Zimbabwe (odds ratio of 2.6 [83]).

Studies Showing an Association between TV and HIV Shedding

Infection with TV has also been associated with increased shedding of HIV in co-infected women. In a group of US women HIV shedding was more frequent in TV-infected women and treatment of TV resulted in reduced shedding at a 3-month visit [84]. Similarly, a study in Kenya found decreased HIV-shedding after treatment of TV [85]. In HSV/HIV-coinfected women in Tanzania, TV was also associated with increased HIV shedding [86].

Epithelial Barrier Disruption by TV

While TV infection can be asymptomatic in some women, in others it can induce dramatic changes in the epithelium. Thus, the vagina and/or cervix can be inflamed and swollen, with erosion of the cervical epithelium and punctate hemorrhages that are referred to as strawberry cervix [87]. The mechanism for these extreme changes in the epithelium have not been fully determined. However, TV binds to epithelial cells *via* adhesins, breaks down the mucous layer and causes epithelial cell apoptosis. TV also promotes inflammatory changes that could contribute to epithelial disruption (see section below).

Adherence of TV to epithelial cells is an important step in causing damage to the epithelium and may involve the lipophosphoglycan and proteins of the parasite membrane as well as host cell lectins [88-91]. TV also secretes proteases and other factors that can be directly cytotoxic to cells that may degrade both the extracellular matrix and anti-microbial proteins [79, 92]. Some of the proteases have been found to cause apoptosis of epithelial cells *in vitro* [93].

To determine possible mechanisms by which TV can increase susceptibility to HIV infection, Guenther *et al.* [94] studied the effects of TV on polarized epithelium *in vitro*. Addition of TV to the apical side of polarized epithelial cell monolayers disrupted epithelial integrity as measured by electrical resistance. This disruption was enough to allow passage of HIV to the basolateral side of the monolayers.

While TV may affect the barrier function provided by epithelial cells, mucins also provide a protective layer that potentially trap HIV and prevent infection. A TV isolate was shown to secrete several proteases [95] and since mucins have a protein backbone these proteases had the capacity to degraded mucins.

Immunity/Inflammation and TV

TV infection can lead to changes in innate and adaptive immunity in the female genital tract that may contribute to increased HIV susceptibility. The interactions of the immune system and TV were recently reviewed [78]. TV infection results in recruitment of immune cells to

the vaginal mucosa and in inflammation. Lymphocytes, including those that express CD4, are increased in swabs taken from mucosa of TV infected women [81, 96, 97]. Neutrophil numbers are also increased in women with TV although this is not known to affect HIV susceptibility [44, 79].

TV infection also results in increased levels of proinflammatory cytokines in genital secretions that could affect HIV susceptibility and shedding in multiple ways (see BV section above). For example, in one study genital IL-1 levels were increased in HIV-infected women with TV infection [98]. In pregnant women with BV, IL-1 and IL-8 levels were increased in women co-infected with TV [44]. Incubation of resting HIV-infected peripheral blood mononuclear cells with TV induced HIV expression and this was dependent on TNF production [94]. Cervical vaginal lavage samples, collected from women with TV, induced the production of IL-8 by cells that express TLR4 suggesting that a TLR4-dependent ligand was present in the genital tract of infected women [99]. Vaginal secretions collected after clearance of infection had less of a stimulatory activity. On the other hand, TV has been reported to be immunosuppressive in some respects as it can induce apoptosis of macrophages by activating them, and can also suppress production of pro-inflammatory cytokines by these cells [100, 101].

Alterations of Bacterial Microbiota Associated with TV

While the above studies suggest that TV by itself could alter susceptibility to HIV infection by disrupting the epithelium or by inducing inflammation, co-incidence of TV and BV is common. In the 2001–2004 National Health and Examination Survey approximately half of TV infected women also had BV [102]. This suggests an additional reason for a TV association with increased susceptibility to HIV; that is, TV could be associated with alterations in bacterial microbiota that in turn influence HIV susceptibility. While most of the studies showing an association between TV and HIV controlled for the presence of BV [81, 82], they did not assess more subtle changes in microbiota. For example, women with low levels of H₂O₂-producing lactobacilli, while not having BV, could be more susceptible to both HIV and TV infection. In fact, in a study of Kenyan women, TV infection was associated with isolation of a lower number of H₂O₂-producing lactobacilli [103]. A study in the US found an association between TV and lower levels of total vaginal lactobacilli as estimated from gram stains [104]. Another possibility is that TV has a direct influence on genital microbiota. In a study of women attending an STD clinic in South Africa, the incidence of TV was positively associated with Nugent score with an incidence of 12% in subjects with a low score of (3 or less) to 33% in those with higher scores [105]. The authors of that study hypothesized that TV may actually lead to greater susceptibility to BV. This makes mechanistic sense since TV alters the vaginal pH which in turn is known to lead to changes in microbiota. By this mechanism, TV could lead to crowding out of H₂O₂-producing lactobacilli.

Concluding Remarks

The female genital tract is protected against pathogenic organisms by the innate and adaptive arms of the immune system, the genital tract microbiota and the epithelium. The perturbations of the vaginal environment caused by BV and/or TV bring about the sorts of immunological and physiological changes that render the lower genital tract more conducive to pathogenic infections such as HIV. These changes include the presence of microbial products (e.g. TLR ligands, SCFAs, and sialidases), the induction of proinflammatory cytokines (e.g. IL-1), the recruitment of target cells to the site of inflammation (e.g. CD4+ cells), and the loss of vaginal epithelium integrity. Given the heterogeneity of microbiota in different individuals, and the diversity in host responses to microorganisms, more work is

needed to determine how these BV- and TV-associated alterations may contribute to HIV susceptibility.

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Fig. (1). Lower Genital Tract Bacterial Microbiota of a group of US Women

Samples were obtained by cervicovaginal lavage from 10 women without BV (left) and 10 with BV (right). Note that BV tends to be polymicrobial while microbiota of non-BV tends to be nearly all lactobacilli. Microbiota was determined by pyrosequencing of the 16S rRNA gene and inputting the sequences into Ribosomal Database 10.

Fig. (2). Lower Genital Tract Bacterial Microbiota of Female Macaques

Samples were provided by Ellen Kersh from ten pigtailed macaques at the Centers for Disease Control and Prevention, by Ron Veazey from nine rhesus macaques from the Tulane National Primate Research Center and by Chris Miller from 11 rhesus macaques at the California National Primate Research Center at UC Davis. Note that the macaque microbiota at these three primate centers has similarities to BV in humans. Microbiota was determined by pyrosequencing of the 16S rRNA gene and inputting the sequencing into Ribosomal Database 10.

Fig. (3). Bacterial Vaginosis Increases Levels of Proinflammatory Cytokines in the Lower Genital Tract

Cervicovaginal lavage samples were obtained from women with no STDs and women with BV and no other STDs. IL-1 β , IL-8, and CXCL9 levels were measured utilizing Cytometric Bead Arrays (BD Biosciences). To detect lactoferrin, custom cytometric bead arrays were made by coupling blank beads with rabbit antibody to lactoferrin. All bead arrays were assayed on a FACS Calibur and levels of cytokines calculated using BD CBA software (BD Biosciences [106]).

Fig. (4). Butyric Acid Induces Production of IL-1

Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were incubated with either LPS (1 μg/ml), butyric acid (BA, 20 mM), acetic acid (AA, 20 mM) or medium only (no stim) for 4 hours in the presence of GolgiPlug (BD Biosciences). Cells were washed and stained with an antibody to CD14. They were then fixed, permeabilized, and stained with an antibody to IL-1 . Events were collected on a FACS Calibur and analyzed using CellQuest software (BD Biosciences). Total PBMCs were gated by light scatter. This figure shows that the CD14-positive monocyte population in PBMCs, but not CD14-negative lymphocytes, are induced by butyric acid to produce IL-1 .

Fig. (5). Butyric Acid, But Not Acetic Acid, Suppresses HIV Infection of Macrophages
 Monocytes were obtained by adherence from PBMCs and differentiated into macrophages by culture in Monocyte Colony Stimulating Factor (M-CSF) for seven days. Left panel: Cells were treated with SCFAs for 16 hours, washed, and infected with HIVBaL for 16 hours. Levels of HIV provirus in cells relative to GAPDH were then determined. Right panel: Cells were infected for 4 hours, washed and treated with SCFAs for 16 hours. After washing, cells were cultured 12 days and culture supernatant HIV p24 levels measured.