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Longitudinal Study of the Biotypes of *Gardnerella vaginalis*

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***Gardnerella vaginalis* is the predominant vaginal microorganism in women with bacterial vaginosis. However, this organism is also frequently isolated from women without signs or symptoms of vaginitis. Earlier studies have not revealed whether certain biotypes of *G. vaginalis* are more often associated with bacterial vaginosis or are more common in women who acquire bacterial vaginosis. We used a typing scheme based on tests for β-galactosidase, hippurate hydrolysis, and lipase, using oleate as a substrate. Of 261 strains tested, the distribution of biotypes observed was as follows: 1, 13%; 2, 9%; 3, 5%; 4, 7%; 5, 41%; 6, 15%; and 8, 10%. Biotype 7 was not observed. The distributions of biotypes from women with and without bacterial vaginosis were found to be significantly different, with the lipase-positive biotypes (biotypes 1, 2, 3, and 4) being more predominant in women with vaginosis (41 versus 23%, $P = 0.003$). Of 40 women with normal vaginal flora at the index visit who remained normal at follow-up, 23 (57%) acquired a new biotype of *G. vaginalis*. By comparison, 90% of the 30 women who developed bacterial vaginosis acquired a new biotype of *G. vaginalis* ($P = 0.003$). Women with bacterial vaginosis at the index visit who were not treated were no more likely than normal women to have a shift in *G. vaginalis* biotype. However, 86% of the 30 women with bacterial vaginosis who were treated with an antibiotic at the index visit acquired a different biotype ($P = 0.04$ compared with the value for untreated women) regardless of treatment success. A trend toward the acquisition of a new biotype was observed among women who had contact with a new sexual partner (81 versus 65%, $P = 0.15$). These data demonstrate that the lipase-positive isolates of *G. vaginalis* are associated with bacterial vaginosis. Women who acquire bacterial vaginosis are more likely to have a shift in biotype than women who had normal flora at the follow-up, suggesting that the *G. vaginalis* isolates recovered from women who develop bacterial vaginosis represent newly acquired strains rather than overgrowth of previously colonizing biotypes.**

Gardnerella vaginalis is the predominant organism isolated from the vaginal fluid of women with bacterial vaginosis (BV) (6a, 19). For more than 25 years, however, this organism's bassociation with the condition has remained poorly understood. In 1955, Gardner and Dukes performed a study in which they isolated *G. vaginalis* from 138 (92%) of 141 women with BV and from 14 (6%) of 232 women without BV and cited the organism as the causative agent of the syndrome, which they named *Haemophilus vaginalis* vaginitis (6). However, since that time, published studies have demonstrated that *G. vaginalis* is often isolated from the vaginal fluid of women who are asymptomatic and lack clinical signs of vaginitis.

McCormack et al., in a study of 466 women without BV, found that 250 (32%) were vaginally colonized by *G. vaginalis* (11) and concluded that *G. vaginalis* is not the sole etiologic agent of bacterial vaginosis. Bump et al. (3, 4), in a study of 52 virginal adolescents, demonstrated that *G. vaginalis* can likewise be isolated from virginal females, showing that *G. vaginalis* is not exclusively sexually transmitted. Since published studies contain conflicting data on the epidemiology of *G. vaginalis*, biotyping of the isolates may provide a better understanding of the pathogenic significance of this organism in relation to bacterial vaginosis.

In 1984, Piot et al. introduced a typing scheme which separated *G. vaginalis* into eight distinct biotypes on the basis of lipase and β-galactosidase activity and hippurate hydrolysis. In a study of 359 vaginal isolates, Piot et al. observed no significant difference in the distributions of *G. vaginalis* biotypes from women with or without BV (15). Benito et al., by using a modified biotyping scheme that incorporated carbohydrate fermentation, found that four biotypes (biotypes 2, 4, 5, and 7) predominated in patients with BV (2). Thus, there exists a disparity among published studies as to whether certain biotypes are associated with BV.

In their study, Piot et al. also examined 48 paired *G. vaginalis* strains isolated from seven women before and after treatment. No change in biotype was observed when cultures were obtained within 7 days. However, when cultures were obtained 2 weeks or more after treatment, 48% of the women were observed to have a change in *G. vaginalis* biotype (15). Piot et al. did not determine whether certain biotypes were more often associated with the acquisition of BV by normal women, whether untreated women with BV have a shift in biotype when BV spontaneously resolves, or whether women who have BV and are treated have a shift in biotype. Finally, no study to date has examined whether the acquisition of a new *G. vaginalis* strain is associated with a new sexual partner.

In this investigation, we used the scheme devised by Piot et al. to determine the biotypes of *G. vaginalis* obtained from women enrolled in a longitudinal study. Isolates obtained at 4-month intervals from women with and without BV and before and after treatment were biotyped. Isolates from women with BV who remained untreated were examined as well.

MATERIALS AND METHODS

Source of isolates. Two hundred sixty-one isolates of *G. vaginalis* were recovered from 124 paired patient visits from sexually active women seeking care at the Seattle-King County Sexually Transmitted Disease Clinic at Harborview Medical Center and at the University of Washington Student Health Clinic in Seattle. The isolates of *G. vaginalis* ob-

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tained at the first visit (index visit) were compared with those obtained at a second visit 4 months later (follow-up visit). Women who presented with symptoms and were diagnosed by clinical signs as having BV were treated either with ampicillin, given at a dosage of 500 mg four times a day for 7 days, or with oral metronidazole, given at a dosage of 500 mg twice a day for 7 days. The patient population had a median age of 23 years and a median of 13 years of education. The median number of lifetime partners was 10 and the median gravidity was 1 with 0 parity.

Diagnosis of BV. Diagnosis of BV was based on the clinical presentation of signs (elevated vaginal pH, vaginal discharge, odor in the presence of 10% KOH, and the presence of clue cells) (1) as well as Gram-stain interpretation. Gram-stain evaluations of each specimen were characterized by the method of Spiegel et al. (20). Each specimen was characterized either as being normal (lactobacillus-predominant flora) or as indicating BV (decreased lactobacilluslike organisms with increased numbers of small gram-negative rods, small gram-positive rods, and gram-positive cocci).

Characterization of *G. vaginalis*. For recovery of *G. vaginalis*, vaginal swab specimens were inoculated onto two HBT (21) (human blood bilayer agar with Tween 80) plates and one 5% sheep blood agar plate (Columbia agar base). One HBT plate and the 5% sheep blood agar plate were incubated aerobically with 5% CO_2 at 37°C for 48 h, and the other HBT plate was incubated under anaerobic conditions with 85% N_2–10% H_2–5% CO_2 at 37°C for 48 h. All *G. vaginalis* isolates were identified by the following criteria (shown to correctly identify greater than 95% of *G. vaginalis* isolates): hemolysis on human blood agar, no hemolysis on sheep blood agar, Gram stain reaction showing typical gram-negative to gram-variable rods, and negative catalase production (10). Several colony types of presumptive *G. vaginalis* were subcultured to ensure isolation of all strains present. After isolation, strains were stocked into litmus milk and stored at −70°C until biotyping was performed.

Biochemical tests. The biotyping scheme was composed of three biochemical tests: hippurate hydrolysis, lipase activity with oleate as a substrate, and β-galactosidase activity. Biotypes were assigned to strains on the basis of the combination of the three test results observed (15). All biochemical assays were completed on *G. vaginalis* isolates obtained from 48-h subcultures incubated at 37°C on chocolate agar.

Lipase activity was determined by a spot test on Whatman no. 2 filter paper (Whatman, Inc., Clifton, N.J.) by using a stock solution of 4-methylumbelliferyl oleate (Sigma, St. Louis, Mo.) prepared in distilled water at a concentration of 110 μmol/ml. This test is a variation of a filter paper sialidase spot test described previously (12). Filter paper strips (60 by 6 mm) containing 40 μl of the oleate substrate solution and 40 μl of buffer composed of 22 mM *N*-octyl-beta-D-glucopyranoside, 12 mM $CaCl_2$, and 125 mM sodium phosphate (pH 7.0) were inoculated with a loopful of bacteria and incubated for 15 min at 37°C. Results were read by examining the strips under a long-wavelength (365 nm), hand-held mineral lamp. A fluorescent blue spot was indicative of positive lipase activity.

β-Galactosidase activity was determined by using a similar filter paper spot test. Stock solutions of 4-methylumbelliferyl-*N*-acetyl-β-D-galactosaminide (Sigma) were prepared in distilled water at a concentration of 110 μmol/ml. Strips of Whatman no. 2 filter paper were immersed in 180 μl of substrate solution and 20 μl of a buffer solution composed of $NaH_2PO_4 \cdot 1H_2O$ (pH 7.0) and were inoculated with a loopful of bacteria. Inoculated filter strips were examined under a long-wavelength (365 nm), hand-held mineral lamp after 15 min of incubation at 37°C. A fluorescent blue spot was indicative of positive β-galactosidase activity.

Hippurate hydrolysis was determined by using a modified rapid test (8). Tubes containing 0.5 ml of hippurate test solution were inoculated with a loopful of bacteria and incubated at 37°C for 2 h. Ninhydrin (200 μl) was added to each tube, and the tubes were reincubated for 10 min at 37°C. Results were read for the appearance of a purple (positive) or a yellow (negative) color.

Statistical analysis. Statistical analysis of data was performed on an IBM computer (by using the program EpiInfo; USD, Inc., Stone Mountain, Ga.). Chi-square analysis of data was done with the Mantel-Haenszel equation.

TABLE 1. Distribution of biotypes among *G. vaginalis* strains isolated from women with or without BV

Biotype no.	No. of isolates (%)[a]	No. positive for BV (%)[b]	No. negative for BV (%)[c]	Odds ratio (95% confidence interval)
1	33 (13)	23 (15)	10 (9)	1.9 (0.8–4.4)
2	23 (9)	16 (11)	7 (6)	1.8 (0.7–5.0)
3	12 (5)	9 (6)	3 (3)	2.3 (0.6–11.2)
4	19 (7)	13 (9)	6 (5)	1.7 (0.6–5.2)
5	108 (41)	56 (38)	52 (46)	0.7 (0.4–1.2)
6	40 (15)	18 (12)	22 (20)	0.6 (0.3–1.2)
8	26 (10)	14 (9)	12 (11)	0.9 (0.4–2.1)

[a] $n = 261$.
[b] $n = 149$.
[c] $n = 112$.

RESULTS

A total of 261 isolates of *G. vaginalis* were recovered: 149 (57%) from women with BV and 112 (43%) from women characterized as normal. Of the eight biotypes described by Piot et al. (15), only seven were observed. Biotypes 5 (41%), 6 (15%), and 1 (13%) were found to be most common (Table 1). Vaginal colonization by multiple biotypes at one time was observed in 48% of the women. A significant difference in the distribution of *G. vaginalis* biotypes from women with BV and women characterized as normal was observed. Biotypes 1, 2, 3, and 4 were found to be more prevalent among women with BV (41 versus 23%; odds ratio, 2.3; 95% confidence interval, 1.3 to 4.0; $P = 0.003$). Biotype 7 was not observed.

The natural history of *G. vaginalis* biotypes determined by a longitudinal study of paired isolates from consecutive visits is displayed in Table 2. Of 70 women with normal vaginal flora at the index visit, 40 women were observed to remain normal and 30 women had acquired BV by the follow-up visit. Of the 40 women who remained normal, 23 (57%) displayed a shift in biotype within the 4-month interval. Among the 30 women who acquired BV, 27 (90%) acquired a new biotype (odds ratio, 6.7; 95% confidence interval, 1.5 to 32.9; $P = 0.003$). Twenty-four women with BV but without symptoms at the index visit were followed without treatment; of these, 17 women had persistent BV and 7 women had normal flora without treatment at follow-up (Table 2). Untreated women with BV were no more likely to have a shift in biotype than women who remained normal at the follow-up visit ($P > 0.6$).

A total of 30 women with symptomatic BV at the index visit were examined for shifts in *G. vaginalis* biotypes before and 2 weeks after treatment with metronidazole or ampicil-

TABLE 2. Natural history of *G. vaginalis* biotypes determined from isolates from paired consecutive visits

Diagnosis[a]	No. (%) of occurrences[b]	No. (%) with same biotype	No. (%) with different biotype	Odds ratio (95% confidence interval) and *P* value[c]
Normal (NL to NL)	40 (43)	17 (43)	23 (57)	
BV acquisition (NL to BV)	30 (32)	3 (10)	27 (90)	6.7 (1.5–32.9), 0.003
Persistent BV (BV to BV)	17 (18)	6 (35)	11 (65)	1.4 (0.36–5.2), 0.6
Resolved spontaneously (BV to NL)	7 (7)	3 (43)	4 (57)	1.0 (0.2–6.6), 1.0

[a] NL, Normal.
[b] $n = 94$.
[c] Comparison with normal women who remained normal.

lin. Twenty-six (86%) of the women had a change in *G. vaginalis* biotype after treatment. Of women with persistent BV, 87% had a shift in biotype, while 86% of the women who became normal with treatment were also found to have a change in *G. vaginalis* biotype (Table 3). Compared with the 24 women with asymptomatic BV who were not treated at the index visit, women who were treated at the index visit were more likely to have a change in biotype at the follow-up visit (odds ratio, 3.9; 95% confidence interval, 1.1 to 14.4; $P = 0.04$).

To determine whether acquisition of a new biotype of *G. vaginalis* was associated with having a new sexual partner, isolates from 74 index and follow-up visits were compared and stratified by whether the female reported having at least one new partner within the preceding 4-month interval. Within the follow-up period, 29 women remained normal, 26 women acquired BV, 13 women had persistent BV when left untreated, and 6 women were observed to revert spontaneously to normal when not treated. Women with a new sex partner were not significantly more likely to acquire a new biotype of *G. vaginalis* than women who did not report a new sexual partner (81 versus 65%; odds ratio, 2.2; $P = 0.15$).

DISCUSSION

In this longitudinal study of *G. vaginalis* biotypes, we demonstrated that four specific biotypes (biotypes 1, 2, 3, and 4) were more often recovered from women with BV. This is in contrast to the observation of Piot et al., who reported that no significant difference in distribution existed among *G. vaginalis* biotypes in association with BV (15). Benito et al., however, did observe a relationship between *G. vaginalis* biotypes and BV, finding biotypes 2, 4, 5, and 7 to be the most prevalent types among women with BV (2). In our study, the four biotypes found to be associated with BV were positive for lipase activity with oleate as the substrate. The increased specificity of the lipase assay used in this study may account for the differences in results observed in our study and those investigations mentioned above. The system devised by Piot et al. and used by Benito et al. tested lipase activity on egg yolk agar, assaying for a wider range of lipolytic activity (7). The lipases of *G. vaginalis* have not been well described, and the role of oleate lipase as a virulence factor in BV has not been studied, although staphylococcal lipases have been demonstrated to be associated with pathogenicity (5).

TABLE 3. Effect of antibiotic treatment on *G. vaginalis* biotypes

Diagnosis of first and follow-up visits[a]	No. of women with a different biotype/total no. of women tested (%)		Odds ratio (95% confidence interval) and *P* value[b]
	Untreated	Treated	
BV, BV	11/17 (65)	14/16 (87)	3.8 (0.7–21.9)
BV, NL	4/7 (57)	12/14 (86)	4.5 (0.6–36.2)
Overall	15/24 (63)	26/30 (86)	3.9 (1.1–14.4), 0.04

[a] NL, Normal.
[b] Comparison of treated versus untreated women with BV.

The overall distribution of *G. vaginalis* biotypes observed in our study is similar to that demonstrated by other investigators who have employed the biotyping scheme of Piot et al. Both Piot et al. (15) and Pandit et al. (13) found biotypes 1 (26 to 60%) and 5 (16 to 35%) to be the most prevalent, with the distribution differing somewhat among the isolates from the United States; Nairobi, Kenya; and Belgium. Benito et al. found biotypes 1 (31%) and 2 (14%) to have the highest frequency in their study of isolates from Spain (2). Scott et al. (16) and Ison et al. (9) found biotypes 1 (51 and 14%), 2 (20 and 34%), and 5 (18 and 30%) to be the most prevalent among women who had BV, but they did not test isolates from asymptomatic carriers. Like these investigators, we found biotypes 1 and 5 at a high frequency. In addition, we found biotype 6 to be a frequent isolate (15%). Piot et al. recovered this biotype in 4 to 9% of the women (15). Slight variations between our data and those obtained by other investigators may be attributable to the increased specificity of the lipase assay used in the current study or may reflect differences due to different geographic locations.

Published studies have contained conflicting data on the possibility of sexual transmission of *G. vaginalis*. Although *G. vaginalis* may be isolated from the vaginas of sexually inactive adolescents, the organism is more often isolated from women who are sexually active (3, 4, 17). More importantly, Piot et al. (15) observed that biotypes isolated from the women with BV were identical to those isolated from the urethras of their sex partners in 11 of 12 cases, whereas Gardner and Dukes (6) and Pheifer et al. (14) recovered *G. vaginalis* from the urethras of 45 (86%) of 47 and 27 (79%) of 34 partners, respectively, of women with BV. In the current study, 32% of the normal women acquired BV during the follow-up period. Of the women who were normal and acquired BV, 90% also acquired a new biotype (odds ratio, 6.7; $P = 0.003$). The high incidence of acquisition of BV may be attributed to the fact that most of the women studied were from a sexually transmitted disease clinic population. While there was a trend toward an association between the acquisition of a new biotype of *G. vaginalis* and a new sex partner, the association was not statistically significant. Ideally, a longitudinal study of the biotypes of *G. vaginalis* from women with and without BV and their male sex partners would clarify the relationship between sexual transmission of *G. vaginalis* and the acquisition of BV.

In our study, 86% of the women colonized by *G. vaginalis* were found to have a different biotype 1 week after completion of a 7-day course of treatment with either metronidazole or ampicillin. Regardless of whether BV resolved, women who were treated were more likely to have a change in *G. vaginalis* biotype than those who were not treated. Such an association is understandable, since ampicillin and the hydroxy metabolite of metronidazole have been demonstrated

to be effective against *G. vaginalis* in vitro (18). In our study, as well as that of Piot et al. (15), women who had persistent BV after treatment were as likely to have a shift in biotype as women who were treated effectively and acquired normal vaginal flora (87 versus 86%).

These data suggest that the presence of *G. vaginalis* after treatment is not associated with treatment failure. The presence of different biotypes following treatment indicates the successful eradication of the original *G. vaginalis* strains and suggests that the new biotypes acquired are a result of reinfection and not persistence. The source of these new biotypes is unclear. Although multiple colony types of *G. vaginalis* were selected for each patient specimen, it is possible that not all of the biotypes present were recovered, since the random selection of colonies does not take into account fluctuation in the numbers of a given biotype present at a specific point in time. Typing isolates from original or replicate plates may be desirable for future studies. In our study, reinfection by the male partner during sexual intercourse after treatment seems unlikely, since partners were protected by condoms during the 2 weeks between visits.

These data suggest that lipase-positive biotypes of *G. vaginalis* are associated with BV and that the acquisition of BV is associated with the acquisition of a new biotype of *G. vaginalis*. Further studies are needed to clarify the association between *G. vaginalis* and BV.

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