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Association of *Mobiluncus curtisii* With Recurrence of Bacterial Vaginosis

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Despite the fact that bacterial vaginosis (BV) is the most prevalent cause of symptomatic vaginal discharge in the United States^{1,2} and is a risk factor for preterm birth³ and acquisition of HIV,⁴ its cause remains unknown and its treatment suboptimal. Not only are initial cure rates of BV poor, but recurrence rates are high.⁵ Debate exists as to whether recurrences are due to relapse or reinfection. However, 1 hypothesis is that relapse may occur secondary to inadequate treatment of BV-associated organisms, either due to resistance of BV-associated organisms to metronidazole or to inadequate penetration of a biofilm community.^{6,7} Anecdotally it is more difficult to eradicate BV in some women compared with others. Recent data substantiates this by the finding that women with more complicated vaginal flora reflected by higher Nugent scores (predominantly because of morphotypes consistent with *Mobiluncus*), are more likely to fail therapy than those with lower scores.⁸ The presence of *Mobiluncus* spp. (*M. curtisii* and *M. mulieris*) in the vagina is highly specific although not sensitive for the diagnosis of BV.^{9,10} Of the two, *M. curtisii* appears to predominate and in vitro is resistant to metronidazole, the most commonly used therapy for BV.¹¹ Therefore, recurrence of BV could be related to failure to clear this organism. Using specimens obtained from a prospective study of the treatment of BV, we determined if there was an association between persistence of *M. curtisii* and recurrence of BV at 65 to 70 days after completion of initial therapy.

Vaginal swabs were collected during a prospective treatment study of BV. Women attending the Jefferson County Health Department STD Clinic with symptomatic BV were enrolled and treated for BV as previously described.⁸ All women received metronidazole for 7 or 14 days plus or minus azithromycin. Subjects were reevaluated at 21, 35 to 40, and 65 to 70 days after beginning the study. Subjects were discontinued from the study upon recurrence of symptomatic BV but were followed if the BV was asymptomatic. Vaginal swabs for Gram stain and polymerase chain reaction (PCR) were collected at baseline and each follow-up visit. For this substudy we included only those women who had *M. curtisii* present at baseline as detected by PCR and who completed 3 follow-up visits for the main study. Recurrence of BV at 65 to 70 days was defined as a Nugent score of 7 to 10 at that follow-up visit regardless of symptoms. Persistence of *M. curtisii* was defined as the organism being present at baseline and at least one of the follow-up visits.

Baseline characteristics of the subsample and larger cohort were compared by the chi-square test for categorical variables and the *t* test for continuous variables. The relationship between the persistence of *M. curtisii* the development of recurrence was examined by the chi-square test. A stratified analysis was used to examine the relationship between *M. curtisii* and

recurrence controlling for treatment and the Breslow-Day statistic was used to test homogeneity of the strata. All analyses were conducted with SAS Ver. 9.1 and a *P* value of <0.05 was deemed statistically significant.

Swabs for PCR were eluted into sterile, deionized water. DNA was extracted from the eluate using Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) following the provided method for isolation of DNA from Gram-negative bacteria. Extracted DNA was rehydrated in 40 µL of the kit's DNA rehydration solution.

Two separate PCR amplifications were performed on the extracted DNA. The first was done with a genus-specific primer set for *Mobiluncus*, Mob-s and Mob-as¹². The second was performed using a species-specific primer set for *M. curtisii*, Mob-V3 and Mob-V4.¹² The reaction volume was 20 or 50 µL consisting of 1 or 2 µL of rehydrated, extracted DNA, 1X buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0 at the rate of 25°C), 1.5 mmol/L MgCl₂, 0.1% Triton X-100), 200 µmol/L dNTPs, 1 µmol/L of each primer, and 0.025 U Taq Polymerase. Amplification conditions were 30 or 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with a final extension of 72°C for 10 minutes. Extracted *M. curtisii* ATCC 35,241 and *M. mulieris* ATCC 35,243 DNA was used for positive amplification controls, and water in place of DNA was used for a negative control. Amplification products were electrophoresed on 2% to 4% agarose gels, stained with ethidium bromide, visualized using UV light, and documented with a digital imager. The amplicons produced were 423 base pairs for the genus-specific and 130 base pairs for the species-specific primers. Samples that exhibited only the 423 bp product were considered positive for *M. mulieris*. Those that exhibited the 423 and 130 bp products were considered positive for *M. curtisii* without the ability to determine the presence or absence of *M. mulieris*.

Gram stains were interpreted according to the method of Nugent et al.¹³ Curved rod morphotypes were presumed to be *Mobiluncus* spp. No attempt was made to differentiate the 2 species of *Mobiluncus* based on Gram stain morphology.

Of the 594 women enrolled in the main study, 100 women met the inclusion criteria for this substudy (presence of *M. curtisii* by PCR at baseline visit and samples for 3 follow-up visits). The major reasons for exclusion were absence of *M. curtisii* at baseline, discontinuation from the study for failed therapy, and loss to follow-up. In total, 400 PCR results were available from these 100 subjects and their 4 visits. Of these visits, 29 were positive for *M. mulieris* and were not included in the comparison of Gram stain versus PCR detection of *M. curtisii*. Three Gram stain results were not available.

There were no significant differences in the majority of baseline characteristics of the women who met the inclusion criteria for this study compared with the other women in the main treatment study including race, age, education level, douching behavior, methods of birth control, and number of sexual partners. Interestingly, more women in the substudy reported a history of STDs (83.0%) compared with the entire sample (71.2%) (*P* = 0.01). The sensitivity and specificity of PCR for *M. curtisii* using the presence of curved rods on Gram stain as gold standard were 91.0% and 73.7%, respectively.

Persistence of *M. curtisii*, defined as present at baseline and at least one other visit was significantly associated with recurrence of BV (*P* < 0.001). Nearly 67.9% (38/56) of women with persistence of *M. curtisii* had recurrence of BV (Nugent score 7–10) compared with 11.4% (5/44) of women with no evidence of *M. curtisii* by PCR after the baseline treatment visit (Table 1). The patterns of *M. curtisii* positivity by study visit are shown in Table 2. The 2 most common patterns of *M. curtisii* persistence were absence of the organism at visits 2 and 3 with detection at visit 4, and presence of the organism at each visit. The former

suggests reinfection whereas the latter suggests that *M. curtisii* was never cleared by the initial therapy. There was no significant difference in the association of *M. curtisii* with recurrent BV when stratified by the addition of azithromycin to the treatment regimen (Table 3).

Adequate treatment for BV remains a goal, not a reality. Initial cure rates range from 50% to 80% and recurrence is common, especially in some women.⁵ Until the etiology of BV is discovered, both in terms of microbiology and transmission, it is unlikely that better therapies will emerge. It has long been appreciated, based on Gram stains of vaginal flora from women with BV, that BV represents a spectrum of abnormalities in the vaginal ecosystem.¹³ Curved rods, felt to represent primarily *Mobiluncus* spp., may be seen in some but not all women with BV.¹³ Because Gram stain detection of *Mobiluncus* is solely dependent on morphotypical analysis, whereas PCR should be highly sensitive and specific, it is not surprising that we did not find complete agreement between Gram stain and PCR for the presence of *Mobiluncus*. We recently showed that higher Nugent scores, reflecting the presence of *Mobiluncus* morphotypes, were significantly associated with failure of initial therapy for BV.⁸ Results from this current study indicate that the presence of *M. curtisii* at baseline along with persistence of this organism as detected by PCR is highly associated with recurrence after initial cure. The reasons for persistence of this organism are unknown and could stem from relapse after therapy or reinfection from a sexual partner. Nevertheless, it appears that the more complex the ecosystem changes associated with BV are, as demonstrated by the presence of anaerobes such as *Mobiluncus*, the more difficult BV is to cure.

In summary, the presence and persistence of *M. curtisii* detected by PCR was found to be highly associated with recurrence of BV. Whether this organism is truly pathogenic or simply a marker for greater disturbances of vaginal flora remains unknown.

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TABLE 1Association of *M. curtisii* With Recurrence of BV

	<i>M. curtisii</i> Positive *	<i>M. curtisii</i> Negative *
Recurrence of BV	38/100 (38.0%)	5/100 (5.0%)
No recurrence of BV	18/100 (18.0%)	39/100 (39.0%)

* *M. curtisii* positive by PCR = present at baseline and at least one other visit; *M. curtisii* negative by PCR = present at 1 visit. $P = 0.001$.

TABLE 2Patterns of *M. curtsii* Prevalence as Detected by PCR at Study Visits

1st Visit (Baseline)	2nd Visit (Day 21)	3rd Visit (Days 35–40)	4th Visit (Days 65–70)	N =
+	–	–	–	44
+	–	–	+	24
+	+	+	+	12
+	–	+	+	7
+	–	+	–	5
+	+	–	+	3
+	–	+	+	3
+	+	–	–	2

TABLE 3Association of *M. curtisii* With Recurrence of BV Stratified by Treatment

	<i>M. curtisii</i> Positive	<i>M. curtisii</i> Negative
Azithromycin (n = 48)		
Recurrence of BV	19/48 (39.6%)	4/78 (8.3%)
No recurrence of BV	6/48 (12.5%)	19/48 (39.6%)
No azithromycin (n = 52)		
Recurrence of BV	19/52 (36.5%)	1/52 (1.9%)
No recurrence of BV	12/52 (23.1%)	20/52 (38.5%)

Breslow-Day test for homogeneity of strata $\chi^2 = 0.34$; $P = 0.56$.