

Quantitative Microbiologic Models for Preterm Delivery

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Preterm delivery (PTD) is the leading cause of infant morbidity and mortality in the United States. An epidemiological association between PTD and various bacteria that are part of the vaginal microflora has been reported. No single bacterial species has been identified as being causally associated with PTD, suggesting a multifactorial etiology. Quantitative microbiologic cultures have been used previously to define normal vaginal microflora in a predictive model. These techniques have been applied to vaginal swab cultures from pregnant women in an effort to develop predictive microbiologic models for PTD. Logistic regression analysis with microbiologic information was performed for various risk groups, and the probability of a PTD was calculated for each subject. Four predictive models were generated by using the quantitative microbiologic data. The area under the curve (AUC) for the receiver operating curves ranged from 0.74 to 0.94, with confidence intervals (CI) ranging from 0.62 to 1. The model for the previous PTD risk group with the highest percentage of PTDs had an AUC of 0.91 (CI, 0.79 to 1). It may be possible to predict PTD by using microbiologic risk factors measured once the gestation period has reached the 20-week time point.

The complex microflora of the human vagina is thought to play an important role in both health and disease. Although a great deal of quantitative and qualitative data describing the vaginal microflora are available, it is only recently that statistical models defining normal vaginal microflora have been developed (33). It has also been possible to predict, on the basis of these quantitative microbiologic models, when the vaginal microflora is abnormal (50, 51). Part of the modeling process is to identify microbial risk factors, such as an abnormal vaginal microflora (i.e., vaginal candidiasis or bacterial vaginosis [BV]), that are associated with a specific outcome. The application of these predictive statistical modeling techniques, using microbiologic data, to other outcomes such as preterm births is appealing because women at risk for adverse pregnancy outcomes might then be identified early during their pregnancies. Early identification of women at high risk for an adverse outcome would allow for interventional therapy to be directed only at such women.

Preterm delivery (PTD) is the leading cause of infant morbidity and mortality in the United States (2, 12, 19). Preterm labor, premature rupture of membranes, and low birth weight have been associated with either specific maternal genital tract infections or an altered vaginal microflora during pregnancy (1, 8, 35, 37, 47, 48). Because bacteria are thought to contribute to PTD, substances of bacterial origin thought to act as mediators of PTD have been sought. Organisms implicated in PTD include specific agents, such as *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *Bacteroides* and yeast species (13, 30, 32, 33, 54, 55). A constellation of organisms representative of those associated with BV have also been cited as having the potential to produce inflammatory mediators, including phospholipases, leading to increased production of prostaglandins

(9, 11, 16, 18, 22, 28, 56). Despite the epidemiological association between PTD and an altered vaginal microflora during pregnancy, the actual mechanism(s) by which preterm labor and delivery occur remains to be identified.

Based on these previous observations, interventional therapy has met with variable success (4, 10, 15, 20, 23, 31, 36). Antimicrobial agents directed at specific organisms, such as *Ureaplasma*, *Chlamydia*, *Gardnerella vaginalis*, and obligately anaerobic microorganisms are generally able to eradicate the organism(s) in question but have little effect on pregnancy outcome. Although certain antibiotic regimens have been shown in some studies to decrease the rate of preterm births in specific groups, such as women with BV, the effects were limited to only a subset of women delivering preterm and were not consistently observed. These observations indicate that, while a variety of abnormal microbiologic findings have been noted in women giving birth after a gestation period of less than 37 weeks, there may also be other risk factors or combinations of risk factors not accounted for by these previous microbiologic and clinical studies.

As the first phase of an ongoing clinical trial designed to determine microbiologic risk factors associated with preterm births, we performed quantitative and qualitative analysis of vaginal microbiologic cultures obtained from 229 pregnant subjects (gestation time point, 20 weeks) that were stratified by risk group. Analysis of the data identified several microbiologic risk factors that were used to construct multivariate predictive microbiologic models for PTD. The second phase of this ongoing trial will be to determine how well such predictive models work in identifying women at risk for preterm births.

MATERIALS AND METHODS

Subjects. Women presenting for their first prenatal care examination at Faculty and Resident Practices, Brigham and Women's Hospital, over a 28-month period were given an enrollment package containing a description of the study. The study nurse identified eligible subjects and approached patients for enroll-

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ment during their routine prenatal visits (each visit occurred when the gestation time had reached between 18 and 22 weeks). The study nurse asked each woman if she was interested in participating in the study and answered any questions regarding the clinical study and sampling procedures. Informed consent was obtained using an institutional review board-approved consent form. Any of the following criteria were considered grounds for exclusion at enrollment: young age (i.e., age of less than 15 years); receipt of antimicrobial therapy within 4 weeks of initial sampling; multiple gestation; previous pregnancy loss (after a gestation period of less than 24 weeks) due to sepsis, cervical cerclage, placenta previa, or Rh isoimmunization; or any other obstetric problem or chronic medical condition predisposing to PTD, including chronic hypertension on medication, pregnancy-induced hypertension, insulin-requiring diabetes, renal disease with a baseline creatinine level of >2.0 , or autoimmune disease requiring steroids. These criteria were designed to exclude women likely to have medically indicated preterm births. Subjects were placed into one of four risk groups: (i) women with no apparent risks, (ii) women with a prior preterm birth, (iii) women with vaginal bleeding during the current pregnancy, and (iv) women with a prior PTD and vaginal bleeding during the current pregnancy. Group sizes for this phase of the study were based on historic data for preterm births of patients at the Faculty and Resident Practice and prior quantitative microbiologic studies designed to detect quantitative differences (i.e., $0.5 \log_{10}$ CFU/g of sample) in bacterial populations of the different groups (44, 45), as gauged by the vaginal swab sampling technique.

Determination of gestational age. Gestational age was determined by using the last menstrual period (LMP) and earliest ultrasound. If the ages determined by using the LMP and the earliest ultrasound were concordant within the defined limits of ultrasound (within 7 days of each other for a gestation period ranging from 0 to 12 weeks, within 10 days of each other for a gestation period ranging from 12 to 20 weeks, within 2 weeks of each other for a gestation period ranging from 20 to 28 weeks, and within 3 weeks of each other for a gestation period of longer than 28 weeks), then the age determined by using the LMP was taken to be correct. If the results were discordant, then the age determined by using the earliest ultrasound was taken to be correct.

Demographic data. Demographic, behavioral, and obstetric data were collected through the use of a questionnaire administered to each subject by the study nurse. Additional demographic and obstetric information was abstracted from each subject's medical record.

Collection of samples for microbiologic analysis. Vaginal swab samples were obtained for laboratory-based microbiologic analysis from enrolled subjects in the antenatal clinic at the Center for Women and Newborn, Brigham and Women's Hospital. The study nurse collected the first vaginal swab samples when the gestational age was between 18 and 22 weeks. To ensure patient confidentiality and to minimize possible bias in the interpretation of laboratory-based tests, all samples were labeled with a numeric code before being sent to the laboratory. The key to the code was kept in a locked file, housed at the Center for Women and Newborn. The code was not linked to the microbiologic data until final outcome data and medical record data were abstracted.

Vaginal swab samples were collected by using the duplicate swab sampling technique for quantitative and qualitative microbiology (7). Briefly, the study nurse inserted two sterile cotton swabs simultaneously into the vaginal vault. The swabs were rotated against the upper vaginal walls to achieve saturation and carefully removed. One swab (the preweighed swab) was returned to a sterile preweighed tube, and the other swab was placed into 10.5 ml of prerduced Amies transport medium without charcoal (PML Microbiologicals, Tualatin, Oreg.). Both swabs were transported to the microbiology laboratory for processing within 2 h. The preweighed swab and tube were reweighed, and the difference between the final weight (of swab and tube together) and the preswab weight (of swab and tube together) was recorded as the sample weight. This method has been validated for obtaining sample weight in previous studies (6, 7, 43). Two additional samples were obtained with sterile cotton swabs: one was used for measuring pH, making a smear for Nugent scoring, and detection of volatile amines, and the other swab was used for *Trichomonas* culture.

Isolation of microorganisms and determination of microbiologic counts. Upon arrival at the microbiology laboratory (within 2 h of sample collection), the swab for microbiologic analysis was placed into an anaerobic chamber for processing. The swab in transport medium was vigorously vortexed to disperse the sample, and serial decimal dilutions (to 10^{-5}) were made in phosphate buffered saline, pH 7.2. A 0.1-ml aliquot of each dilution was plated onto enrichment or selective agar media, and the aliquot was spread over the surface of the medium with a sterile plastic tube. The media used for recovery of obligate anaerobes included BMB (prereduced brucella base blood agar containing 5% sheep blood, hemin, and menadione) and BMB containing 5% laked sheep blood, 100 μg of kanamycin per ml, and 7.5 μg of vancomycin per ml (PML Microbiologicals).

Facultative organisms were isolated with tryptic soy base-5% sheep blood agar, MacConkey's agar, and mannitol-salt agar (all from PML Microbiologicals). Fastidious organisms were isolated on chocolate agar (PML Microbiologicals) (42, 44-46). A7 medium was used for the isolation of *Mycoplasma* and *Ureaplasma* species (Northeast Laboratory, Waterville, Maine). *Trichomonas* organisms were detected with Diamond's medium (PML Microbiologicals). Testing for *C. trachomatis* is routinely performed for all antenatal subjects in the Clinical Microbiology Laboratory at the Brigham and Women's Hospital with the use of a nucleic acid probe system (GenProbe, San Diego, Calif.).

Identification of microorganisms. Following incubation under appropriate atmospheric conditions, colonies were enumerated on the various agar media and individual colony types were selected for identification based on colony morphology and Gram stain results. A variety of phenotypic methods, including long-chain fatty acid analysis, short-chain fatty acid analysis, and traditional biochemical testing, were used for identification purposes as described previously (14, 43-46). All counts were recorded as the numbers of \log_{10} CFU per gram of sample.

Determination of H_2O_2 production. Following identification, the numerically predominant *Lactobacillus* isolate for each sample was tested for production of hydrogen peroxide (H_2O_2) by a modified method described previously (17). The medium included DeMan-Rogosa-Sharpe agar (for detection of lactobacilli; Difco Laboratories, Detroit, Mich.) as the base, and tetra methyl benzidine hydrochloride hydrate (0.25 mg/ml; Sigma, St. Louis, Mo.) and horseradish peroxidase (0.02 mg/liter; Sigma) were added. Each isolate was inoculated onto the prepared plates and incubated in an anaerobic chamber for 2 to 3 days. The plates were removed from the chamber and exposed to ambient air. H_2O_2 was determined to be present if blue pigment was visible in and around the H_2O_2 -producing colonies.

Diagnosis of BV. The results of testing by the clinical criteria of Amsel (pH >4.5 , presence of an amine odor upon addition of 10% KOH to the swab sample, presence of clue cells detected microscopically in wet mounts, and presence of a thin, homogenous vaginal discharge) were recorded for all subjects (2). In addition, the Nugent Gram stain scoring system was used (this system scores the numbers of lactobacilli, gram-negative and gram-variable rods [i.e., *Prevotella* and *G. vaginalis*], and curved gram-variable rods [i.e., *Mobiluncus*]) (26, 40, 41, 53). Unstained smears were obtained at each visit and sent to the microbiology laboratory for Gram staining and scored by two independent evaluators (A.B.O. and M.L.D.).

Statistical methods. All data were maintained in relational databases created with commercial software (Access 97; Microsoft), with a common sample number linking demographic and outcome data to microbiologic data. Possible microbiologic risk factors were identified by using Fisher's exact test for observational data and the *t* test for independent means as well as for correlation coefficients for numeric data (Statistica; Softstat, Tulsa, Okla.). Predictive models were generated by stepwise logistic regression analysis. Cross-validations were performed by the leave-one-out procedure.

RESULTS

Demographic information. A total of 231 women were enrolled in the study; however, 2 subjects were not included in the analysis (1 subject was excluded following an in utero death due to fetal cytomegalovirus infection before the gestation period reached 24 weeks, and the sample from 1 subject was lost in transit to the laboratory). Of the 229 remaining subjects, 48 (21%) were in the vaginal-bleeding-only risk group, 37 (16%) were in the previous-PTD risk group, 19 (8%) were in the previous-PTD-and-vaginal-bleeding risk group, and 125 (55%) were in the no-risk group. A total of 36 (15.7%) spontaneous preterm births occurred during this study. Women in the risk group with a prior preterm birth history (with or without vaginal bleeding) had the highest rate (33.9%) of PTD during this study period (for women with no obvious risk factors, the PTD rate was 8.9% [$P < 0.001$], and for women with only vaginal bleeding during this pregnancy, the PTD rate was 14.5% [$P < 0.03$]). The distribution of births by gestational age is shown in Table 1. Subjects ranged in age from less than 20 years old (0.5% of subjects) to more than 40 years old (4.5% of

TABLE 1. Gestational age at birth

Week	No. of births observed
21.....	1
24.....	1
28.....	1
31.....	1
33.....	4
34.....	9
35.....	6
36.....	13
37.....	21
38.....	43
39.....	56
40.....	50
41.....	20
42.....	2
43.....	1
Total	229

subjects), with 95% of subjects being between the ages of 20 and 39. The mean age for women delivering at less than 37 weeks was 29.8 years (standard deviation, ± 5.7 years), and for women delivering at term, the mean age was 29.9 years (standard deviation, ± 5.6 years). Approximately 55% of subjects had private medical insurance, with the rest being either self-insured or uninsured. A comparison of pregnancy outcomes by insurance status indicated that women who are uninsured or self-insured have a higher rate of preterm births than do women with insurance (22.2 versus 10.7%; *P* < 0.03). No significant differences in outcome based on ethnic group, Nugent score, parity (nulliparous, multiparous, and prima gravida), smoking history, or alcohol history were noted.

Descriptive microbiologic information. A comparison of frequencies of isolation and mean counts for the major genera

comprising the vaginal microflora is shown in Table 2. For the 229 subjects included in this study, 2,059 isolates representing 113 phenotypes were characterized. The mean values for total anaerobic bacterium counts, total aerobic bacterium counts, vaginal pH, and Nugent scores were similar for women delivering at less than 37 weeks and for those delivering at term. No statistically significant differences between the PTD group and the term delivery group were noted in frequencies of isolation and mean counts for the major microbiologic categories. The frequencies of isolation and mean total counts for *G. vaginalis*, *Prevotella* spp., *Peptostreptococcus* spp., *Mycoplasma hominis*, and *Ureaplasma urealyticum* were similar for women delivering at less than 37 weeks and those delivering at term. *Trichomonas* sp. strains were isolated from two subjects (0.1%). Neither subject delivered preterm.

Evaluation of the multiple isolates of lactobacilli encountered during this study revealed that women who were simultaneously harboring strains that produced H₂O₂ and strains that did not produce H₂O₂ were much more likely to have PTDs. While no statistical difference was noted for women harboring either strains that produced H₂O₂ or strains that did not produce H₂O₂ (10 of 33 women who had PTDs and 59 of 154 women who had at-term deliveries [*P* > 0.40] for strains producing H₂O₂; 5 of 33 women who had PTDs and 42 of 154 women who had at-term deliveries [*P* > 0.18] for strains not producing H₂O₂), it was noted that a significantly higher percentage of women harboring both strain types had PTDs (15 of 33 women versus 39 of 154 women [*P* < 0.03]). (Results of testing for H₂O₂ production were not available for 3 women who had PTDs or for 39 women who had at-term deliveries.) In addition, the total number of H₂O₂-producing lactobacilli was significantly lower in women who had PTDs than in those who had at-term deliveries (mean ± standard error of the mean,

TABLE 2. Vaginal microflora summary^a

Organism(s)	Value for gestation time of:			
	<37 weeks		≥37 weeks	
	Frequency of isolation ^b	No. of organisms ^c	Frequency of isolation ^b	No. of organisms
All anaerobes		8.33 ± 1.47		8.38 ± 1.57
All aerobes		7.24 ± 2.21		7.12 ± 2.29
<i>Corynebacterium</i> spp.	26/36	4.50 ± 0.87	212/193	4.66 ± 0.88
<i>Lactobacillus</i> sp. ^d	75/36	7.82 ± 1.43	377/193	7.99 ± 1.30
H ₂ O ₂ positive	40/36	7.69 ± 1.50	195/193	8.25 ± 1.21 ^e
H ₂ O ₂ negative	25/36	7.49 ± 1.42	119/193	7.60 ± 1.36
<i>Gardnerella vaginalis</i>	13/36	8.78 ± 1.30	55/193	8.90 ± 1.28
<i>Prevotella</i> spp.	44/36	5.98 ± 1.62	177/193	5.72 ± 1.80
<i>Prevotella bivia</i>	8/36	5.83 ± 1.50	42/193	5.53 ± 1.50
<i>Peptostreptococcus</i> spp.	32/36	5.30 ± 1.40	134/193	5.44 ± 1.43
<i>Peptostreptococcus assacharolyticus</i>	16/36	5.23 ± 1.35	55/193	5.05 ± 1.26
<i>Peptostreptococcus magnus</i>	10/36	5.23 ± 1.40	44/193	5.41 ± 1.55
<i>Peptostreptococcus prevotii</i>	2/36	5.70 ± 0.40	23/193	6.13 ± 1.08
<i>Staphylococcus</i> spp.	43/36	4.14 ± 0.69	226/193	4.18 ± 0.70
<i>Streptococcus</i> spp.	37/36	5.62 ± 1.66	111/193	4.99 ± 1.32
<i>Mycoplasma hominis</i>	7/36	6.15 ± 0.81	36/193	6.03 ± 1.02
<i>Ureaplasma urealyticum</i>	5/36	6.26 ± 0.88	45/193	5.86 ± 0.73

^a Vaginal pH and Nugent scores were as follows (values are means ± standard deviations): for a gestation period of <37 weeks, 4.76 ± 0.78 and 2.8 ± 2.75, respectively, and for a gestation period of >37 weeks, 4.74 ± 0.79 and 2.45 ± 2.60, respectively.

^b Values are numbers of observations/numbers of subjects.

^c Values are log₁₀ CFU/gram of sample ± standard deviations.

^d A total of 10 strains in the preterm group and 63 strains in the term group were not characterized for H₂O₂ production.

^e *P* < 0.01 when compared to value for gestation period of <37 weeks.

	gestborn	ph	totalan	totalae	maxlacto	maxh2o2	maxprevo
gestborn	1.0000						
ph	-0.0003	1.0000					
totalan	0.0065	0.0731	1.0000				
totalae	0.0391	0.3026*	-0.2351*	1.0000			
maxlacto	-0.0416	-0.3020*	0.0938	-0.1730*	1.0000		
maxh2o2	-0.0686	-0.1179	0.5535*	-0.2490*	0.2221*	1.0000	
maxprevo	-0.0169	0.4814*	0.1817*	0.4904*	-0.2459*	-0.1394	1.0000
ecoli	-0.0307	0.2532*	-0.0196	0.0787	-0.1144	0.0667	0.1830*
maxpepto	-0.0137	0.2828*	0.1161	0.3070*	-0.1673*	-0.1502*	0.5614*
maxstrep	-0.0369	0.3705*	0.0005	0.1901*	-0.3108*	-0.0700	0.2433*
maxstaph	-0.0670	0.1279	-0.1656*	0.2799*	-0.2428*	-0.0856	0.2031*
maxcoryn	-0.0354	0.1600*	0.0161	0.2305*	-0.1498*	-0.0990	0.3529*
gardner	-0.0012	0.3098*	0.1555*	0.5873*	-0.2396*	-0.0298	0.6091*
avgtot	-0.0724	0.4377*	0.1769*	0.4949*	-0.3395*	-0.2180*	0.6619*

	ecoli	maxpepto	maxstrep	maxstaph	maxcoryn	gardner	avgtot
ecoli	1.0000						
maxpepto	-0.0287	1.0000					
maxstrep	0.1164	0.1350*	1.0000				
maxstaph	0.1911*	0.1506*	0.2238*	1.0000			
maxcoryn	-0.0376	0.3749*	0.1923*	0.3378*	1.0000		
gardner	0.0049	0.3667*	0.0524	0.1422*	0.1550*	1.0000	
avgtot	0.1170	0.3763*	0.1863*	0.1492*	0.1687*	0.7282*	1.0000

FIG. 1. Correlation matrix for all subjects. *, significance at P value of <0.05 . Abbreviations: gestborn, gestational age at birth; ph, pH; totalan, all anaerobes; totalae, all aerobes; maxlacto, maximum count of *Lactobacillus* spp.; maxh2o2, maximum count of H_2O_2 -producing *Lactobacillus* spp.; maxprevo, maximum count of *Prevotella* spp.; ecoli, *Escherichia coli* count; maxpepto, maximum count of *Peptostreptococcus* spp.; maxstrep, maximum count of *Streptococcus* spp.; maxstaph, maximum count of *Staphylococcus* spp.; maxcoryn, maximum count of *Corynebacterium* spp.; gardner, *Gardnerella* sp. count; avgtot, average total number of bacteria.

7.69 ± 0.24 versus 8.25 ± 0.09 \log_{10} CFU/g of sample; $P < 0.01$).

Predictive statistical model for PTD. A correlation matrix containing the major microbiologic members of the vaginal microflora was constructed as a preliminary step in developing statistical models for preterm birth (Fig. 1). There was a strong overall correlation between the total aerobic bacterium counts and the average total bacterium count (avgtot) ($r = 0.49$; $P < 0.01$). An even stronger correlation existed between these two variables for the subset of women delivering after a gestation period of less than 37 weeks ($r = 0.62$; $P < 0.01$). The maximum counts of H_2O_2 -producing lactobacilli and total anaerobic bacteria were noted to show a significant correlation ($r = 0.55$; $P < 0.01$). An even stronger correlation existed between these two variables for women delivering after a gestation period of less than 37 weeks ($r = 0.61$; $P < 0.01$). Vaginal pH levels had a positive correlation with the total aerobic counts ($r = 0.30$; $P < 0.01$) and a negative correlation with the total counts for *Lactobacillus* spp. ($r = -0.30$; $P < 0.01$) (as the pH decreased, the count of lactobacilli increased).

For women in different risk groups, we examined whether PTD might be related to somewhat different microbial profiles. Therefore, we considered separate models for women with and without prior PTDs.

For the women who had had a prior PTD, the rate of preterm birth was 37.8% for the period of this study. For these women, we considered two models: model 1 included women who had no vaginal bleeding during the current pregnancy, and model 2 included women with or without vaginal bleeding during the current pregnancy.

On the other hand, for women who had not previously had a PTD, the rate of preterm birth for women with vaginal

bleeding was 14.5% and for women with no obvious risk factor the rate of preterm birth was 8.9%. Hence, these two groups were combined and models 3 and 4 were instead constructed according to the presence or absence of H_2O_2 -positive *Lactobacillus* spp., as follows: model 3 included women with H_2O_2 -positive *Lactobacillus* spp. present, and model 4 included women from whom H_2O_2 -positive *Lactobacillus* spp. were absent.

Models 2, 3, and 4 are based on mutually exclusive groups of patients. Model 1 is a subset of Model 2 that focused on women without vaginal bleeding during the current pregnancy. A total of 206 (90%) of the 229 study subjects were evaluated by one of the models. Subjects with missing data were not evaluated.

For the previous-PTD risk group, the model was constructed as shown below, using numerous iterations to find the best fit. If p is taken to be the probability of PTD, then logit (p) can be calculated as follows:

$$\log[p/(1-p)] = a_0 + a_1 \times (\text{risk factor 1}) + a_2 \times (\text{risk factor 2}) \dots + a_n \times (\text{risk factor } n) \quad (1)$$

where $a_1 \dots a_n$ are estimated regression coefficients. If the maximum counts for H_2O_2 -producing lactobacilli were not available, then the maximum counts for *Lactobacillus* spp. were used in the regression.

One measure for the overall performance of the predictive logistic regression model is the area under the receiver operating curve (AUC). Subjects with predicted probability values above a specified threshold value were considered to be likely to have a PTD during the current pregnancy. The threshold probability was chosen so that the sensitivity and specificity values of the predictive model could be optimized.

TABLE 3. Regression models by risk groups

Risk group characteristic(s)	m/n ^a	Regression model	AUC	CI for AUC
Previous PTD but no vaginal bleeding	9/28	logit (p) = 20.86 - 1.33 × pH - 2.6 × total anaerobic count + 0.58 × maximum H ₂ O ₂ -positive <i>Lactobacillus</i> sp. count + 0.70 × maximum <i>Peptostreptococcus</i> sp. count	0.91	0.79–1.00
Previous PTD with or without vaginal bleeding	11/32	logit (p) = 27.35 - 3.27 × pH + 1.92 × both ^b - 2.22 × total anaerobic bacterium count + 0.64 × maximum H ₂ O ₂ -positive <i>Lactobacillus</i> sp. count + 0.22 × maximum <i>Peptostreptococcus</i> sp. count + 0.21 × <i>Gardnerella</i> sp. count - 0.32 × maximum <i>Staphylococcus</i> sp. count	0.82	0.65–0.98
Bleeding or no obvious risks; H ₂ O ₂ -positive lactobacilli present	15/139	logit (p) = 2.05 - 0.75 × total anaerobic bacterium count - 0.48 × total aerobic bacterium count + 0.29 × maximum H ₂ O ₂ -positive <i>Lactobacillus</i> sp. count + 0.21 × maximum <i>Staphylococcus</i> sp. count + 0.27 × maximum <i>Corynebacterium</i> sp. count + 0.34 × maximum <i>Gardnerella</i> sp. count	0.74	0.62–0.87
Bleeding or no obvious risks; H ₂ O ₂ -positive lactobacilli absent	2/35	logit (p) = -81.14 - 0.45 × total aerobic bacterium count + 8.72 × maximum <i>Lactobacillus</i> sp. count + 0.74 × maximum <i>Streptococcus</i> sp. count	0.94	0.81–1.00

^a m, number of PTD cases; n, number of subjects with all covariates.

^b both, indicator variable for having both vaginal bleeding and previous PTD.

For women with a prior PTD but without vaginal bleeding during the current pregnancy, the logistic regression function for model 1 is as follows: logit (p) = 20.86 - 1.32 × pH - 2.61 × total anaerobic count + 0.58 × maximum count of H₂O₂-positive lactobacilli + 0.70 × maximum count of *Peptostreptococcus* spp. Using this model, the predicted probability (p) is a number between 0 and 1. The area under the curve (AUC) has a value of 0.94, with a confidence interval (CI) of 0.79 to 1. Cross-validation by the leave-one-out procedure was performed, and the results were relatively stable. Comparison of the results predicted on the basis of the logistic regression model to the outcomes actually observed indicated that the predictive logistic model with a cutoff probability of 0.3 yielded a sensitivity of 88.9% and a specificity of 89.5% in predicting which subjects would have PTDs. The microbiologic risk factors included in the regression equation and the AUC measures for the four models are shown in Table 3. Predicted probabilities and actual outcomes for model 1 based on subjects with a prior PTD are shown in Table 4.

DISCUSSION

Previous studies conducted in our laboratory have extensively characterized the vaginal microflora of overtly healthy women (42, 44, 45). Based on the accumulated data from these earlier studies, a predictive model for normal vaginal microflora was constructed and tested (34). Prospective use of this model to predict an abnormal vaginal microflora has proven to be a practical application of these methods (49–52). The present study was an attempt to apply similar microbiologic and statistical methods to the prediction of preterm births, based on quantitative analysis of the vaginal microflora analysis after 20 weeks of gestation.

Our previous studies have demonstrated that, for any given bacterial phenotype, the range of concentrations varies widely from subject to subject as well as for the same subject sampled longitudinally. This may account for the difficulty in correlating the presence or absence of any specific species or total bacterial counts with an outcome such as preterm birth. It was noted during the development of a statistical model for normal vaginal microflora that the most predictive models included mul-

iple bacterial risk factors. In the present study, we elected to use only microbiologic data or vaginal pH levels as risk factors for predicting PTD. Although a variety of demographic data for each subject were available, only the risk group was con-

TABLE 4. Probability calculations and actual outcomes for women with a previous PTD^a

Subject	Probability of PTD	Outcome
1	0.0002	Term
2	0.0155	Term
3	0.0393	Term
4	0.0559	Term
5	0.0655	Term
6	0.0734	Term
7	0.0759	Term
8	0.0779	Term
9	0.0835	Term
10	0.0853	Term
11	0.1102	Term
12	0.1149	Term
13	0.1336	Term
14	0.1766	Term
15	0.2378	Term
16	0.2378	Term
17	0.2881	Term
18	0.6093	Term* ^b
19	0.9414	Term* ^b
20	0.1988	Preterm* ^b
21	0.3204	Preterm
22	0.5244	Preterm
23	0.5604	Preterm
24	0.6063	Preterm
25	0.7116	Preterm
26	0.7941	Preterm
27	0.9221	Preterm
28	0.9422	Preterm

^a Data shown are for 28 of 36 subjects without any missing data.

^b *, probability calculation with a cutoff of 0.3 varies from actual outcome. All three subjects thus denoted had total anaerobic counts that were significantly higher than those observed for the rest of the group (P < 0.034).

sidered during construction of the predictive models. This approach allows for the use of observed microbiologic data in the absence of other confounding variables, such as a history of smoking and alcohol use, that have been suggested to influence pregnancy outcome. Subsequent statistical modeling has included both demographic data (such as age, ethnic group, insurance status, history of smoking and/or alcohol use), and parity. While these models may be more complete in that they use all of the available data, the AUC for these models is not significantly better than that obtained when only microbiologic data are used (unpublished data).

Among the organisms most commonly present as vaginal microflora are members of the genus *Lactobacillus*. *Lactobacillus acidophilus* is often reported to be the member of this genus most commonly isolated from vaginal secretions. Most of these previous reports have relied exclusively on conventional methods of phenotyping. However, recent studies using 16S rRNA-based ribotyping indicate that *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasserii* are the species most commonly present as part of the vaginal microflora (3). The role of lactobacilli within the vaginal environment is generally regarded as beneficial, with the production of lactic acid being responsible, in part, for the low pH of the vagina. This low pH is inhibitory for many potentially pathogenic organisms that also colonize the vaginal environment in low concentrations. More recently, it has been reported that the production of H₂O₂ by some members of the genus *Lactobacillus* is also associated with a healthy vaginal environment (3, 17, 25, 27). The inhibitory effect in vitro of H₂O₂ for organisms not producing catalase is well known. With regard to the vaginal microflora, the inhibitory effect of H₂O₂ for *G. vaginalis* has been documented, although the effect of H₂O₂ within the vaginal environment for other non-catalase-producing species, including anaerobic streptococci and other obligate anaerobes, is not known. Women with BV have decreased colonization rates for H₂O₂-producing strains of lactobacilli and increased colonization rates for non-H₂O₂-producing strains. It has also been reported that non-H₂O₂-producing strains of lactobacilli are present as part of the normal vaginal microflora (21, 24, 25, 29). In many women, both H₂O₂- and non-H₂O₂-producing strains can be isolated. While the presence or absence of H₂O₂- and non-H₂O₂-producing strains has been reported with respect to pregnancy outcome, this is the first attempt to use such information to develop a predictive model for PTD. Of particular note during the construction of the predictive models is the central role for both the type and total numbers of lactobacilli present as part of the vaginal microflora. The observation that women harboring both H₂O₂-producing and non-H₂O₂-producing *Lactobacillus* strains are at increased risk for PTD is a clear indication that this genus is an important microbiologic risk factor for predicting outcome. Although the characterization of lactobacilli in this initial phase of the study included only genus designation and H₂O₂ production, our continuing study includes characterization of isolates by restriction fragment length polymorphism and pulsed-field gel electrophoresis analyses, species-level identification by 16S ribosomal DNA-based sequencing, and testing for the presence of lactocin genes by highly specific molecular probes. Further characterization of these strains by molecular techniques may provide additional useful information.

Many gram-positive organisms produce small peptides that are antibacterial in nature, variously called bacteriocins, lantibiotics, and lactocins. In the case of lactobacilli, such substances are often directed at suppressing growth of closely related species. It has been reported, for example, that a bacteriocin produced by *L. acidophilus* is directed at inhibition of *Lactobacillus debrueckii*, a closely related species (5). The characteristics of these small peptide inhibitors produced by lactobacilli are remarkably similar among gram-positive species and often contain posttranslational modifications of specific amino acids (38). Some of these compounds, such as lactocin S, can act as both bacteriocin and as cytolysin (38). While lactobacilli may be useful markers for a healthy vaginal environment, the possibility that specific strains or combinations of strains are harmful should not be ignored, particularly in light of their ability to produce substances capable of inhibiting other normal vaginal microflora organisms. The elevated vaginal pH associated with pregnancy, coupled with the presence of multiple strains of lactobacilli, may provide an ideal environment for production of bacteriocins. It has also been reported that a bacteriocin similar to those produced by lactobacilli is capable of inducing cellular injury (39) via the generation of superoxide anions. Among the genera present as part of the vaginal microflora, *Lactobacillus* is almost unique in that it has never been explored as a possible mediator of tissue inflammation.

The reliability of the models defined here to prospectively predict PTD is currently being evaluated in an ongoing clinical trial. If the models are able to prospectively predict preterm births reliably, the ability to use targeted interventional therapy only for those women at risk for preterm birth becomes a genuine possibility. The ability to construct these models using only microbiologic data suggests a primary role for bacteria in the etiology of preterm births. Understanding the mechanisms by which bacteria contribute to PTD is the basis for our continued research efforts.

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