ELSEVIER

Assessment of cervical antibody concentrations fails to enhance the value of cervical length as a predictor of preterm delivery

Rodney K. Edwards, MD, MS,[a] Ronald J. Ferguson, PhD,[a] Jonathan J. Shuster, PhD,[b] Douglas Theriaque, MS,[b] Susan Gentry, MSN, ARNP,[a] Patrick Duff, MD[a]

Departments of Obstetrics and Gynecology[a] and Statistics,[b] University of Florida College of Medicine, Gainesville, Fla

Objective: The purpose of this study was to determine if cervical fluid antibody concentrations can enhance the value of cervical length in predicting risk of preterm delivery.
Study design: We obtained cervical fluid samples with preweighed cellulose wicks from a prospective cohort of women 23 to 32 weeks' gestation with signs and symptoms of preterm labor and intact membranes. Total immunoglobulin A and G (IgA and IgG) concentrations were determined by enzyme-linked immunosorbent assay. Bacterial vaginosis was diagnosed by Gram stain, and cervical length was measured with endovaginal ultrasound.
Results: For subjects with term (n = 77) and preterm (n = 24) deliveries, median IgA and IgG concentrations were 736 vs 643 μg/mL (P = .33) and 1528 vs 1769 μg/mL (P = .85). For subjects with normal flora (n = 71), intermediate flora (n = 14), and bacterial vaginosis (n = 16), median IgA and IgG concentrations were 717, 624, and 774 μg/mL (P = .90) and 1383, 1553, and 2731 μg/mL (P = .02). In a forward stepwise logistic regression model, cervical length was the only factor associated with preterm delivery ($P < .001$).
Conclusion: Measuring the concentrations of IgA and IgG in cervical fluid does not enhance the value of cervical length in predicting risk of preterm delivery.
© 2005 Elsevier Inc. All rights reserved.

More than 12% of all births occur before 37 weeks' gestation.[1] The complications of preterm birth cause more than 70% of the deaths of nonanomalous neonates, and are responsible for the majority of morbidity suffered by such infants.[2]

Bacterial vaginosis is a polymicrobial infection that is an established risk factor for preterm delivery.[3-6] Bacterial vaginosis is characterized by an increase in vaginal pH, an amine odor, and an increased bacterial count. In this disorder, there is a replacement of the naturally predominant bacteria in the vaginal flora (*Lactobacillus* sp.) by a complex mixture of anaerobic and facultative anaerobic bacteria.[7,8] The mechanism by which bacterial vaginosis causes preterm delivery is thought to be due to ascension of organisms through the cervix and into the uterus.[9]

Bacteria colonize the vagina both in patients with normal vaginal flora and in those with bacterial vaginosis. Because the uterus normally is sterile, it seems logical that factors at the level of the cervix are involved in maintaining the sterility of the upper genital tract. The length of the cervix, measured with endovaginal ultrasound examination, is inversely related to the risk of preterm delivery.[10-12] However, the reason for this relationship is not well defined.

Cervical mucus displays antimicrobial properties in both nonpregnant and pregnant women.[13-15] Secretory antibodies, or immunoglobulins, function to perform immune exclusion at mucosal surfaces. Immunoglobulin A (IgA) and immunoglobulin G (IgG) are the predominant classes of antibody recoverable from the endocervix.[16] These antibodies act to exclude vaginal microorganisms from the upper genital tract. We speculated that the reason for the inverse relationship between cervical length and preterm delivery was that the cervix functions as an "immune tunnel" between the vagina and the uterine cavity. A shorter cervical canal and/or a lower concentration of antibodies within this cervical canal might allow organisms easier access to the uterine cavity. The objective of this study was to determine whether measuring the concentrations of antibodies in cervical fluid could further refine the risk of preterm delivery associated with cervical length.

Material and methods

We performed a prospective observational cohort study at Shands Hospital at the University of Florida. Subjects were enrolled from September 2001 to March 2003. Women were eligible for inclusion in this cohort if they presented to the labor and delivery unit for evaluation of uterine contractions, had a singleton gestation, and were between 23 and 32 weeks' gestation. Exclusion criteria included ruptured membranes, human immunodeficiency virus (HIV) infection, placenta previa or abruption, cervical dilation ≥ 3 cm, or administration of antibiotics within the preceding 2 weeks. In addition, women with clinical chorioamnionitis at the time of presentation were excluded. We defined clinical chorioamnionitis as a temperature $\geq 38.0°C$ and 1 or more of: maternal heart rate >100 beats per minute, fetal baseline heart rate >160 beats per minute, or uterine tenderness. The study was conducted in accordance with the guidelines established by the University of Florida Health Center Institutional Review Board.

After informed written and oral consent was obtained, and before any manipulation of the cervix, a sterile speculum examination was performed on each subject. Cervical fluid samples were obtained by placing a cellulose acetate wick (UniWick™, Whatman, Clifton,

NJ) approximately 5 mm into the cervical os with a sterile forceps, taking care not to contact the vagina or vaginal fluid. The wick was left in place for approximately 60 seconds and removed. Wicks were stored in preweighed microcentrifuge tubes before sample collection. After sample collection, the wicks were returned to the same tube, taken immediately to the laboratory, postweighed, and stored at –80°C until fluid was eluted from the wicks.

At the time of the speculum examination, a swab from the vaginal fornix was obtained from each subject and used to prepare a slide for Gram stain for diagnosis of bacterial vaginosis using the method of Nugent et al.[17] The speculum then was removed, and the cervical length was measured with a real-time endovaginal ultrasound examination, according to the method described by Iams et al.[10]

For elution of fluid from the cervical wicks, 600 µL of elution buffer was added to each microcentrifuge tube containing a wick. This solution was composed of phosphate-buffered saline (PBS) with 2% Triton X-100, 0.2 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 µmol/L leupeptin, 1 µg/mL aprotinin, and 3.25 µmol/L bestatin. Fluid was extracted on ice for 30 minutes, vortexing 3 to 4 times. Contents of the microcentrifuge tubes then were transferred into centrifuge filter tubes (VectaSpin 3™; Whatman, Maidstone, England) and centrifuged at 4°C for 12 minutes at 3000g. Supernatants were removed and used for analysis. Using known concentrations of antibodies absorbed by wicks (Cappel purified human secretory IgA and purified human IgG; ICN Pharmaceuticals, Aurora, Ohio), we determined that this elution process recovered >95% of antibody from the wicks (data not shown). We assumed that cervical fluid has a density equivalent to water (1 g/mL). Because we knew the difference in weight before and after sample collection and used a constant volume of elution buffer, true concentrations of antibodies in the cervical fluid could be calculated.

IgA and IgG enzyme-linked immunosorbent assays (ELISAs) were performed using modifications of the techniques described by Quesnel et al.[16] Briefly, for the IgA ELISA, Fisher*brand* high binding 96-well plates (Fisher Scientific, Pittsburgh, Pa) were coated with 2.5 µg/mL of Cappel goat affinity-purified antibody to human IgA (α-chain; ICN Pharmaceuticals) in carbonate buffer pH 9.6 as the primary antibody and a 1:2000 dilution of Cappel peroxidase-conjugated sheep affinity-purified antibody to human IgA (α-chain; ICN Pharmaceuticals) in PBS, pH 7.4, with 0.05% Tween 20 (PBS-T) as the secondary antibody solution. For the IgG ELISA, Fisher*brand* high binding 96-well plates were coated with 5 µg/mL of Cappel goat affinity-purified antibody to human IgG Fc (ICN Pharmaceuticals) in carbonate buffer pH 9.6 as the primary antibody, and a 1:8000 dilution of Cappel

Table I Demographic data of study population

	Preterm (n = 24)	Term (n = 77)
Age (y)	23.5 (20.5-29.5)	22 (19-30)
Race		
White	54.2	63.6
Black	41.7	31.2
Other	4.2	5.2
Parous	29.2	44.2
Previous preterm delivery	33.3	16.9
Smoker	16.7	18.2
Gestational age (wk)		
Enrollment	28 (27-30)	29 (27-31)
Delivery	35 (33-36)	38 (37-39)
Diabetes		
No	100	94.8
Gestational	0	2.6
Pregestational	0	2.6
Antenatal infections		
Bacterial vaginosis	12.5	14.3
Chlamydia	0	7.8
Gonorrhea	0	1.3
Trichomoniasis	12.5	7.8
Syphilis	0	0
Urinary infection	20.8	15.6

Data are presented as median (interquartile range) or proportion of n.

Table II Cervical fluid antibody concentrations and cervical length stratified by term or preterm delivery

Factor	Term (n = 77)	Preterm (n = 24)	P value
IgA (µg/mL)	736 (397-1195)	643 (388-782)	.33
IgG (µg/mL)	1528 (1033-2497)	1769 (980-2954)	.85
Cervical length (mm)	35 (28-42)	22.5 (16-30)	< .001

Data are presented as median (interquartile range).

peroxidase-conjugated goat IgG fraction to human IgG Fab (ICN Pharmaceuticals) in PBS-T, 1% gelatin (Sigma-Aldrich, St. Louis, Mo) as the secondary antibody solution.

Demographic data and relevant maternal medical information were abstracted from each subject's medical record. The primary outcome was delivery before 37 weeks' gestation. Forward stepwise logistic regression[18] was used to evaluate the association of preterm delivery with cervical length, cervical fluid IgA concentration, cervical fluid IgG concentration, and Nugent score. Univariate comparisons of timing of delivery and Nugent score-based groups are also presented using the Kruskal-Wallis test.[19] All tests of statistical significance were two-tailed, and used an alpha of .05 to define statistical significance.

Power calculations for regression models are complex. We took the simplified approach of looking only at total IgA in conjunction with the BV factor in order to obtain an approximate estimate of the adequacy of 200 patients. Assuming that 24% of the subjects would have a preterm delivery, 40% would have BV, 50% of patients without BV would have IgA concentrations above the median, and 20% without BV would have IgA concentrations above the median, with that number of subjects, we would have had a power of .66 for the BV factor, .97 for the total IgA factor, and .80 for the interaction factor. Note that the test for each will become more efficient with the addition of demographic covariates to the model.

Results

During the study period, 137 patients were enrolled in the study, and complete delivery information was available for 134. For this analysis, another 33 subjects were excluded because of visible staining of the cervical wick by blood. Therefore, 101 subjects were analyzed. Demographic data are displayed in Table I. Gestational age at study entry did not differ between groups. As would be expected, a higher proportion of subjects in the group delivering preterm had a previous preterm birth.

Median cervical fluid antibody concentrations stratified by term or preterm delivery and by vaginal Gram stain result, respectively, are shown in Tables II and III. The rate of preterm delivery in this cohort was 24% (24 of 101 subjects). Four of the subjects delivering before 37 weeks had "indicated" preterm births caused by maternal disease. Results were not substantially different if these subjects were included in the term group, rather than the preterm group (data not shown).

In a forward stepwise logistic regression model with preterm birth as the dependent variable and cervical length, IgA, IgG, and Nugent score (2 variables to include 3 levels) as independent variables, cervical length was highly correlated with preterm delivery ($P < .001$). After controlling for cervical length, none of the other variables were significantly prognostic for preterm delivery (residual overall $P = .75$). From this model, it was estimated that a 1-mm decrease in cervical length was associated with a 10% (95% CI 5%-15%) increase in the risk of preterm delivery, and that a 10-mm decrease in cervical length was associated with a 157% (95% CI 57%-319%) increase in the risk of a preterm birth. In other words, a patient with a cervical length of 20 mm is estimated to have 2.57 times the risk of preterm delivery as a patient whose cervical length was 30 mm.

Comment

Previous reports have established that cervical length is inversely related to the risk of preterm delivery.[10-12] Results from our cohort are consistent with the findings from those studies, and confirm the value of cervical length in predicting the risk of preterm delivery.

Table III Cervical fluid antibody concentrations and cervical length stratified by vaginal Gram stain result

Factor	Normal flora (n = 71)	Intermediate flora (n = 14)	Bacterial vaginosis (n = 16)	P value
IgA (µg/mL)	717 (379-1136)	624 (466-893)	774 (420-1229)	.90*
IgG (µg/mL)	1383 (981-2212)	1553 (1033-3058)	2731 (1639-4631)	.02*
Cervical length (mm)	35 (26-42)	29.5 (24-41)	25 (21-35.5)	.10*

Data are presented as median (interquartile range). The Nugent score ranges correlating with normal flora, intermediate flora, and bacterial vaginosis, respectively, are 0-3, 4-6, and 7-10.

* *P* values reported in this table are for the overall comparison between the normal flora, intermediate flora, and bacterial vaginosis groups.

However, the reason for the association between short cervix and preterm delivery remains undefined.

The relationship may be due to biomechanical factors such as uterine contractions and the inherent tensile strength of the cervical tissues. However, bacterial vaginosis, an established risk factor for preterm delivery, is thought to cause preterm delivery because of ascension of organisms through the cervix and into the uterus.[9] Furthermore, clinical or subclinical infection of the uterus is thought to play a major role in the majority of cases of spontaneous preterm birth, particularly those that occur before 34 weeks.[20] These associations are not explained by biomechanical factors.

Measuring the concentrations of total IgA and IgG in the cervical fluid, as we did in this study, does not appear to enhance the predictive value of cervical length. The cohort that we reported in this manuscript is rather small, and type II errors are possible. In the univariate analysis, IgG concentrations were increased in the setting of bacterial vaginosis. This finding may be caused by the fact that bacterial vaginosis causes not only an inflammatory response in the host but also an immune response. However, although IgG concentrations were increased in the setting of bacterial vaginosis in univariate analysis, there was no trend indicating that the concentration of either antibody in the cervical fluid correlates with preterm delivery. Therefore, we think that clinically significant differences would be unlikely, even if we had enrolled the planned number of subjects.

The concentrations of immunoglobulins that we measured from the cervical fluid of subjects in this study were similar to the concentrations reported by Quesnel et al, using the same collection method in nonpregnant women.[16] Kutteh and Franklin, using a different method for collection, reported concentrations of IgA and IgG in cervical fluid samples from pregnant women that were 2 to 5 times lower than those that we found.[21] However, they also reported concentrations of these antibodies in the cervical fluid of nonpregnant women that were lower than the values that they measured in pregnant subjects, and therefore, differed even more with the nonpregnant values reported by Quesnel et al. Although we did not control for microscopic amounts of blood in samples, such contamination (if present) would not have affected the results for IgA because serum levels of IgA in pregnant women are similar to those in cervical mucus.[22] Serum levels of IgG are higher than cervical fluid levels of this antibody, and microscopic blood contamination could elevate a given subject's concentration of this antibody.

Despite the negative findings in this study, the concept that cervical length is inversely related to preterm delivery because the cervix functions as an "immune tunnel" between the vagina and the uterine cavity may still, at least partially, explain the relationship. It may be that antigen-specific, rather than total, immunoglobulins are important in modulating the risk of preterm delivery related to cervical length. One group of investigators has evaluated, with mixed results, IgA directed against a certain toxin of *Gardnerella vaginalis*, a bacterial vaginosis-related organism.[23,24] Alternatively, factors associated with the innate, rather than the adaptive, immune response may warrant investigation. Other authors have demonstrated the presence of such factors as secretory leukoprotease inhibitor, lysozyme, lactoferrin, and defensins in cervical mucus at concentrations sufficient for antimicrobial activity.[25] Further work measuring these and other factors may help to explain the pathophysiology of preterm delivery and the mechanism of the relationship between short cervix and this outcome. Such antimicrobial constituents of the innate immune response might become candidate agents for treatment or prevention of preterm delivery.

References

1. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML. Births: final data for 2002. Natl Vital Stat Rep 2003;52:1-113.
2. Arias E, MacDorman MF, Strobino DM, Guyer B. Annual summary of vital statistics—2002. Pediatrics 2003;112:1215-30.
3. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and *Chlamydia trachomatis* infection with adverse pregnancy outcome. JAMA 1986;256:1899-903.
4. McDonald HM, O'Loughlin JA, Jolley P, Vigneswaran R, McDonald PJ. Prenatal microbiological risk factors associated with preterm birth. BJOG 1992;99:190-6.

5. Meis PJ, Goldenberg RL, Mercer B, Moawad A, Das A, McNellis D, et al. The preterm prediction study: significance of vaginal infections. Am J Obstet Gynecol 1995;173:1231-5.

6. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, et al. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. N Engl J Med 1995;333:1737-42.

7. Spiegel CA. Bacterial vaginosis. Clin Microbiol Rev 1991;4:485-502.

8. Krohn MA, Hillier SL, Lee ML, Rabe LK, Eschenbach DA. Vaginal *Bacteroides* species are associated with an increased rate of preterm delivery among women in preterm labor. J Infect Dis 1991;164:88-93.

9. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 1988;319:972-8.

10. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. National Institute of Child Health and Human Development Maternal Fetal Medicine Unit Network. N Engl J Med 1996;334:567-72.

11. Berghella V, Tolosa JE, Kuhlman K, Weiner S, Bolognese RJ, Wapner RJ. Cervical ultrasonography compared with manual examination as a predictor of preterm delivery. Am J Obstet Gynecol 1997;177:723-30.

12. Colombo DF, Iams JD. Cervical length and preterm labor. Clin Obstet Gynecol 2000;43:735-45.

13. Zuckerman H, Kahana A, Carmel S. Antibacterial activity of human cervical mucus. Gynecol Invest 1975;6:265-71.

14. Eggert-Kruse W, Botz I, Pohl S, Rohr G, Strowitzki T. Antimicrobial activity of human cervical mucus. Hum Reprod 2000;15:778-84.

15. Hein M, Helmig RB, Schonheyder HC, Ganz T, Uldbjerg N. An in vitro study of antibacterial properties of the cervical mucus plug in pregnancy. Am J Obstet Gynecol 2001;185:586-92.

16. Quesnel A, Cu-Uvin S, Murphy D, Ashley RL, Flanigan T, Neutra MR. Comparative analysis of methods for collection and measurement of immunoglobulins in cervical and vaginal secretions of women. J Immunol Methods 1997;202:153-61.

17. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J Clin Microbiol 1991;29:297-301.

18. Bartolucci AA, Fraser MD. Comparative step-up and composite tests for selecting prognostic indicators associated with survival. Biometr J 1977;19:437-48.

19. Lehmann EL. Nonparametrics: statistical methods based on ranks. San Francisco: Holden-Day; 1975.

20. Gomez R, Romero R, Edwin SS, David C. Pathogenesis of preterm labor and preterm premature rupture of membranes associated with intraamniotic infection. Infect Dis Clin N Am 1997;11:135-76.

21. Kutteh WH, Franklin RD. Quantification of immunoglobulins and cytokines in human cervical mucus during each trimester of pregnancy. Am J Obstet Gynecol 2001;184:865-74.

22. Briese V, Seyfarth M, Brock J. Serum concentration of secretory IgA during pregnancy and in gynaecological diseases affecting glands and mucosas. Zentralbl Gynakol 1991;113:987-94.

23. Cauci S, Thorsen P, Schendel DE, Bremmelgaard A, Quadrifoglio F, Guaschino S. Determination of immunoglobulin A against *Gardnerella vaginalis* hemolysin, sialidase, and prolidase activities in vaginal fluid: implications for adverse pregnancy outcomes. J Clin Microbiol 2003;41:435-8.

24. Cauci S, Hitti J, Noonan C, Agnew K, Quadrifoglio F, Hillier SL, et al. Vaginal hydrolytic enzymes, immunoglobulin A against *Gardnerella vaginalis* toxin, and risk of early preterm birth among women in preterm labor with bacterial vaginosis or intermediate flora. Am J Obstet Gynecol 2002;187:877-81.

25. Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T. Antimicrobial factors in the cervical mucus plug. Am J Obstet Gynecol 2002;187:137-44.