Anaerobe 14 (2008) 256–260

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical Microbiology

Prevotella bivia as a source of lipopolysaccharide in the vagina

Alla Aroutcheva [a,*], Zaodung Ling [b], Sebastian Faro [c]

[a] *Rush University Medical School/Stroger Hospital of Cook County, Division of Infectious Diseases, 1901 W. Harrison Str (#LL 715), Chicago, IL, USA*
[b] *Rush University College of Pharmacology, 1653 W Congress Pkw, Chicago, IL, USA*
[c] *Women's Hospital of Texas, 7400 Fannin, Houston, TX, USA*

ARTICLE INFO

Article history:
Received 7 December 2007
Received in revised form 28 July 2008
Accepted 22 August 2008
Available online 20 September 2008

Keywords:
Bacterial vaginosis
Prevotella bivia
Gardnerella vaginalis
E. coli lipopolysaccharides
Dopamine neurons
Rat model

ABSTRACT

Objectives: To compare vaginal lipopolysaccharides (LPS) concentrations between patients with and without bacterial vaginosis (BV), to evaluate the correlation between *Prevotella bivia* colonization density and LPS concentration, and to determine the impact of LPS on loss of dopamine neurons (DA).

Methods: Vaginal washes obtained from patients with ($n = 43$) and without ($n = 59$) BV were tested for quantity of *P. bivia* cells using quantitative PCR and for concentrations of LPS using the Limulus Amebocyte Lysate gel clot method. *Prevotella bivia*, *Gardnerella vaginalis* and *Escherichia coli* sonicated cell extracts were also tested for LPS production. DA neuron cells obtained from embryonic day (E) 14.5 pregnant rats were exposed to fluid from eight vaginal washes; tyrosine hydrolase immunoreactive staining was applied for visualization and cell counts.

Results: The median LPS concentrations were dramatically higher among patients who had symptoms of BV compared to those who did not have symptoms (3235.0 vs 46.4 EU/ml, respectively, $P < 0.001$); patients who had BV also had much higher colonization densities of *P. bivia* (0.06 ± 0.36 vs 5.4 ± 2.2 $\log_{10}$ CFU/ml, respectively, $P < 0.001$).
Prevotella bivia cell lysates resulted in a higher LPS concentration ($10{,}713.0 \pm 306.6$ EU/ml) than either *E. coli* (4679.0 ± 585.3 EU/ml) or *G. vaginalis* (0.07 ± 0.01 EU/ml of LPS).
The loss of DA neuron was 20–27% in cultures treated with vaginal washes from BV-negative patients and 58–97% in cultures treated with vaginal washes from patients with BV.

Conclusion: P. bivia produces high LPS concentration, which may create a toxic vaginal environment that damages DA neurons.

1. Introduction

The gram-negative bacterial vaginosis (BV)-associated anaerobe, *Prevotella bivia*, inhabits the lower genital tract and has the propensity for adherence to and invasion of human cervix epithelial cells [1]. Anaerobic bacteria were isolated in 91% and 18% of BV-positive and BV- negative patients, respectively [2]; *Prevotella bivia* and other *Prevotella* spp. represented 44% of all anaerobes isolated in the BV-positive group. Evaluation of BV with gram-stained vaginal slides showed that *Prevotella* and *Gardnerella vaginalis* counts correlated strongly with the Nugent score [3].

It has been shown that an increased rate of premature labor and preterm delivery [1–3] occurs among women with *P. bivia* at a concentration of $>10^4$ bacteria/ml of vaginal fluid [4]. An experimental rabbit model also confirmed an association between *P. bivia* and preterm birth, increased level of TNF-α, and chronic intrauterine and fetal infection [5]. A persistent intrauterine inflammatory state which can occur in chronic conditions such as BV suggests that preterm delivery may follow chronic exposure to organisms, perhaps with resulting fetal brain damage [6,7].

Prevotella bivia and other *Prevotella* species contain endotoxin, lipopolysaccharides (LPS), on their outer membranes; LPS is the most potent antigenic component of the gram-negative bacterial cell wall. Some species of *Prevotella* produce LPS that is much more potent in inducing a rapid platelet response than the LPS found in *Escherichia coli* and *Salmonella typhimurium* [8].

Exposure to a low dose of the bacteriotoxin, LPS, during a critical window of vulnerability of fetal development led to the birth of rat pups with fewer than normal dopamine (DA) neurons [9,10].

Since BV results in a relatively high concentration of LPS-producing *P. bivia* in vaginal fluid, the aim of this study was to determine the correlation between the quantity of *P. bivia* and the level of LPS in the vagina of patients with BV. We also investigated whether LPS presented in the vaginal fluid of patients with BV is toxic for dopamine neurons.

* Corresponding author. Tel.: +1 312 864 7053; fax: +1 312 864 9522.
E-mail address: aaroutch@rush.edu (A. Aroutcheva).

1075-9964/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2008.08.002

2. Material and methods

2.1. Vaginal wash

A total of 102 vaginal washes (43 from patients with BV and 59 from patients without BV) were obtained from women of reproductive age visiting the Women's Hospital of Texas, Houston, TX. The samples were studied for the level of endotoxin, lipopolysaccharides (LPS) and the quantity of *P. bivia* in the vagina. All patients enrolled into the study signed written informed consent. The study had approval from the Woman's Hospital of Texas IRB committee.

BV was diagnosed based on clinical characteristics (Amsel criteria) [11] and on interpretation of gram-stained slides, as described by Nugent et al. [12]. Vaginal washes were conducted by instilling 3 ml of sterile non-pyrogenic saline into the vagina and aspirating the fluid with a sterile syringe. The process was repeated using the saline aliquot three times. The sample was maintained at −20 °C and shipped to the laboratory packed in dry ice. Before testing washes were centrifuged at 11,000 × *g* for 15 min to separate cells. The supernatant was used for LPS detection and the cell pellet was used for extraction of DNA.

2.2. LPS determination

LPS was measured using the Limulus Amebocyte Lysate (LAL) (Cape Cod Inc., East Falmouth, MA) in Pyrotell gel clot method according to the manufacturer's instructions. Briefly, the supernatant from vaginal washes and bacterial cell walls was diluted two fold using endotoxin free water and tubes (Cape Cod Inc.). Then 0.1 ml (v/v) of LAL reagent was added to each tube. After 60 min of incubation in a water bath at 37 °C, the tubes were removed and gently inverted. If gel had formed and remained intact at the bottom of the tube, the test was evaluated as positive. The concentration of endotoxin in the sample was calculated by multiplying the dilution factor on LAL-positive tests; the lowest concentration that can be determined using the gel clot technique is 0.03 LPS EU/ml. *Escherichia coli* endotoxin (Cape Cod Inc.) was used as a control.

To compare LPS production by gram-negative vaginal microorganisms, four strains of each microorganisms, *P. bivia*, *G. vaginalis* and *E. coli* were tested. *Prevotella bivia* and *G. vaginalis* were isolated from study patients in the Microbiology Laboratory of Women's Hospital of Texas and four vaginal strains of *E. coli* were selected from our culture collection; no patients with vaginal *E coli* were found in the current study group.

Cells were harvested from agar plates after 24 h of incubation and suspended in endotoxin free water (Cape Cod Inc.) to achieve 0.5 McFarland turbidity, which corresponds to 1.5×10^8 CFU/ml. The suspension was then diluted 10-fold to a concentration of 10^7 CFU/ml. Cell walls were disrupted using sonication (15 s 3 times). Lysate was separated from cell debris by centrifugation and then tested for LPS concentration.

2.3. DNA isolation

The vaginal wash pellets containing squamous epithelial and bacterial cells were used for DNA isolation. DNA was isolated using the Bactozol kit (Molecular Research Center, Inc., Cincinnati, OH). Briefly, cell suspension was treated with 0.1 ml of 1× Bactozyme and incubated at 50 °C for 60 min. After lysis was achieved, we used DNAzol and polyacrylamide to isolate DNA. The DNA was precipitated by adding 100% ethanol.

The total DNA concentration at A_{260} and purity at ratio A_{260}/A_{280} was measured using a Beckman DU-600 spectrophotometer.

2.4. Quantitative PCR

For sequencing and amplification of the *Prevotella bivia* 16S rRNA gene, the following primers were used: forward: 5′-AGG GAT AAC CCA CCG AAA GTT GGA-3′, reverse: 5′-TAA ATC CGG ATA ACG CCC GAA CCT-3′. Selected sequences were analyzed for homology using the GenBank database with BLAST web site (National Center of Biotechnology Information, National Institute of Health, Bethesda, MD). All primers were obtained from IDT, Coralville, IA.

Quantitative PCR was performed using a Light Cycler 143 (Roche, Indianapolis, IN) and SYBR green, as a fluorescent dye (Takara Bio Inc., Otsu Shiga, Japan). After 95 °C 30 s of denaturation, the shuttle PCR protocol involved 35 cycles of 95 °C for 5 s and 60 °C for 20 s. Purified genomic DNA from *P. bivia* ATCC 29303 was used to generate a standard curve consisting of 10^3 to 10^7 copies of DNA [13]. The bacterial counts represent the total amount in 1 ml of vaginal wash. This was obtained by determining the total DNA isolated from the wash samples times the number of bacteria in 50 ng of DNA.

2.5. Dopamine neuron cell culture bioassay

Eight vaginal washes collected from five patients with BV and three patients with a normal vaginal ecosystem were employed for the DA toxicity study. Vaginal washes collected from women with BV contained $\log_{10}$ 6.64 ± 0.73 of *P. bivia* cells/ml and LPS at a level of 8072 EU/ml (2 patients) and of 5030 EU/ml (1 patient); two other washes contained $\log_{10}$6.10 of *P. bivia* cells/ml and 2048 EU/ml of LPS. Three patients without BV had no *P. bivia* in the vagina and LPS at a level of 0 EU/ml (2 patients) and of 16 EU/ml (1 patient). DA neuron tissue culture was obtained from embryonic day (E) 14.5 pregnant rats. Procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) of Rush University. The protocol for this in vitro study has been published elsewhere [14]. The number of tyrosine hydrolase (TH) immunoreactive cells assessed using the procedure is described in detail elsewhere [15]. Briefly, the single cell suspension was prepared from mesencephalic tissue using sequential incubation with trypsin and DNase/trypsin inhibitors. The tissue was triturated into a single cell suspension in 3 ml of complete media and plated into the 48-well plate. The plate was incubated for 24 h to stabilize DA neurons. The following day, 20% of the media was replaced with vaginal wash fluid for an additional 72 h incubation. The plate was washed with PBS, fixed with 4% formaldehyde, and prepared for immunochemistry with (TH) stain, the traditional marker for DA neurons. The plate was incubated with blocking solution containing 0.25% Triton X and normal horse serum for 1 h. Without washing, mouse anti-rat TH antibody (Immunostar, Stillwater, MN) was added and the plate was incubated at 4 °C overnight. The endogenous peroxidase activity was eliminated using a1-h incubation with 0.1% periodate. The immunohistochemical procedure was continued by using a biotinylated horse anti-mouse IgG (0.5%; Vector Laboratories, Burlingame, CA) for 1 h and peroxidase conjugated avidin–biotin complex (Vector Laboratories) for 1 h. The TH immunoreactive cells were visualized using 0.05% 3,3′-diaminobenzidine (DAB), 0.5% nickel sulfate, and 0.003% H_2O_2 in I/A solution (10 mM imidazole/50 mM sodium acetate).

2.6. Statistical analysis

Categorical variables were tested by performing the chi-square test. We graphically displayed the distribution of *P. bivia* colonization density and the LPS concentration as a scatter plot and evaluated the correlation of these two factors by calculating Spearman's rho. For all analyses we used a *P*-value of 0.01 to indicate statistical