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Journal

Journal of Medical Microbiology, 63(Pt_7)

ISSN

0022-2615

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Publication Date

2014-07-01

DOI

10.1099/jmm.0.073080-0

Peer reviewed

Characterization of culturable vaginal *Lactobacillus* species among women with and without bacterial vaginosis from the United States and India: a cross-sectional study

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Received 10 January 2014
Accepted 13 May 2014

Lactobacillus species play an integral part in the health of the vaginal microbiota. We compared vaginal *Lactobacillus* species in women from India and the USA with and without bacterial vaginosis (BV). Between July 2009 and November 2010, a cross-sectional study was conducted among 40 women attending a women's health clinic in Mysore, India, and a sexually transmitted diseases clinic in San Francisco, USA. Women were diagnosed with BV using Amsel's criteria and the Nugent score. *Lactobacillus* 16S rDNA was sequenced to speciate the cultured isolates. Ten Indian and 10 US women without BV were compared with an equal number of women with BV. Lactobacilli were isolated from all healthy women, but from only 10 % of Indian and 50 % of US women with BV. 16S rDNA from 164 *Lactobacillus* colonies was sequenced from healthy women (126 colonies) and women with BV (38 colonies). Seven cultivable *Lactobacillus* species were isolated from 11 Indian women and nine species from 15 US women. The majority of *Lactobacillus* species among Indian women were *L. crispatus* (25 .0%), *L. jensenii* (25.0 %) and *L. reuteri* (16.7 %). Among US women, *L. crispatus* (32.0 %), *L. jensenii* (20.0 %) and *L. coleohominis* (12.0 %) predominated. *L. jensenii* and *L. crispatus* dominated the vaginal flora of healthy Indian and US women. Indian women appeared to have a higher percentage of obligate heterofermentative species, suggesting the need for a larger degree of metabolic flexibility and a more challenging vaginal environment.

INTRODUCTION

It is well accepted that *Lactobacillus* species are a critical component of the vaginal microflora of healthy women. These Gram-positive rods have been shown to have a protective effect against overgrowth by pathogenic micro-organisms (Thomas, 1928). While studies have demonstrated that the vaginal microflora is dominated by four *Lactobacillus* species, *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners*, there is substantial heterogeneity among different human populations (Pavlova *et al.*, 2002).

Abbreviations: BV, bacterial vaginosis; PHRII, Public Health Research Institute of India.

Studies in the USA, Europe and Japan have shown that women are predominantly colonized by obligate homofermentative lactobacilli that produce only lactic acid (Giorgi *et al.*, 1987; Antonio *et al.*, 1999; Song *et al.*, 1999; Vásquez *et al.*, 2002; Martín *et al.*, 2008). Pavlova *et al.* (2002) found that the vaginal microbiota was dominated by *L. crispatus*, *L. jensenii* and *L. gasseri* in seven countries, while the microbiota of women from several low- and middle-income countries more often included heterofermentative *Lactobacillus* species such as *L. vaginalis*, *L. fermentum* and *L. rhamnosus*. Other studies have also shown greater numbers of heterofermentative vaginal *Lactobacillus* among women from various developing countries (Ocana *et al.*, 1999; Jin *et al.*, 2007;

Martinez *et al.*, 2008; Garg *et al.*, 2009; Damelin *et al.*, 2011; Kazi *et al.*, 2012), compared with women in the USA and Europe (Antonio *et al.*, 1999; Vásquez *et al.*, 2002). The aim of this study was to characterize vaginal lactobacilli species in US and Indian women with and without bacterial vaginosis (BV).

METHODS

Between July 2009 and November 2010, participants were recruited from a reproductive health clinic in Mysore, India, and a sexually transmitted diseases clinic in San Francisco, USA. Study eligibility criteria included age 18–35 years, sexually active and the ability to provide informed consent. Women who were pregnant, menstruating or with a diagnosis of any reproductive tract infections except BV, who had used antibiotics in the prior 30 days or who had a history of taking probiotics were excluded. The study was approved by the institutional review boards of the University of California, Berkeley, and the Public Health Research Institute of India (PHRII).

All participants underwent a physical examination and biological specimens were collected to detect reproductive tract infections. The diagnosis of BV was initially based on the criteria of Amsel *et al.* (1983). Three vaginal swab specimens were obtained from the posterior fornix of the vagina. The first swab was used to measure the vaginal pH, with the swab smeared onto a microscope slide and then placed in a tube containing four drops of normal saline for wet-mount preparation. The remaining two swabs were transferred to BBL Port-A-Cul tubes (Becton Dickinson) and stored at −20 °C until further use. Microscope slides were Gram stained to obtain the Nugent score (Nugent *et al.*, 1991). The vaginal flora was defined as 'healthy' if the score was 0–3; 'intermediate' at 4–6; and 'BV' at ⩾7. Only women diagnosed as 'healthy' or 'BV' were included in the analyses. Lactobacilli were characterized by type of glucose fermentation as obligate homofermentative, facultative heterofermentative or obligate heterofermentative species according to the review of taxonomic information gathered by Felis & Dellaglio (2007).

In India, the two swabs were transported to the PHRII laboratory. The swabs were plated on Rogosa and sheep blood agar (HiMedia Laboratories) within 12 h of collection, and incubated for 24–48 h at 37 °C in 5 % CO_2 anaerobic jars containing AnaeroPacks (Mitsubishi). In the USA, the swabs were cultured on Rogosa (BD Diagnostics) and Columbia blood agar plates (Hardy Diagnostics) and incubated as described above. Only plates containing 10–200 c.f.u. were further analysed. Up to 10 colonies were randomly selected from each plate. Gram-positive rods were further evaluated.

A modified version of the bead-beating method was used to extract bacterial DNA for PCR analysis (Rantakokko-Jalava & Jalava, 2002). *Lactobacillus* 16S rDNA PCR amplification was carried out according to a reported protocol with the primers published previously (Martinez-Freijo *et al.*, 1998). *Escherichia coli* ATCC 25922 DNA was used as a positive control for the 16S rDNA. PCR products were purified using an AxyPrep kit (Axygen) or GeneJET gel extraction kit (Fermentas) in India and using ExoSAP-IT (USB) in the USA, and sequenced on a 3730 DNA analyser (Applied Biosystems) at the DNA Sequencing Facility of the University of California, Berkeley, USA, and at SciGenom or Eurofins Genomics, India. Genus and species were determined by the criteria of 98 % sequence identity for species and 95 % sequence identity for genus (Bäckhed *et al.*, 2005).

Data analysis. Data were analysed using SAS version 9.3 (SAS Institute). A Fisher's exact test was performed to test the relationship between BV status and the presence of any lactobacilli and specific *Lactobacillus* species. Two-tailed P values <0.05 were considered statistically significant. Only samples with confirmed BLAST identity ⩾95 % and match length ⩾500 bp were included in the analysis. Three BV-positive vaginal samples from India were excluded because they did not meet these criteria.

RESULTS AND DISCUSSION

Vaginal samples were obtained from 40 reproductive-age women. Twenty women were healthy (10 Indian, 10 US) and 20 had BV (10 Indian, 10 US). All 20 US women reported inconsistent condom use, and none used hormonal contraceptives. Enrolled US participants were seen at the clinic to screen for eligibility to participate in a larger research study, and were not regular patients of a sexually transmitted diseases clinic with symptoms or complaints. A majority of the Indian women reported tubal ligation for birth control (n=16; 80 %) and the remainder used no contraceptive methods. The primary reason for Indian participants to attend the reproductive health clinic was a routine health check-up.

Of the 40 women, 37 (17 Indian and 20 US) had *Lactobacillus* 16S rDNA with a sequence match length of ⩾500 bp and BLAST identity ⩾95 %. Of these 37 women, 26 (70 %; 11 Indian and 15 US) had cultivable *Lactobacillus* colonies. Lactobacilli were isolated from all healthy Indian and US women, but from only one Indian and five US women with BV. *Lactobacillus* 16S rDNA was successfully sequenced for 164 isolates. Unsurprisingly, 126 isolates were from healthy women (58 isolates from India and 68 from the USA) and only 38 isolates were from women with BV (10 isolates from India and 28 from the USA). Overall, 76.8 % of cultivable *Lactobacillus* species were obtained

Table 1. Distribution of colonizing *Lactobacillus* species by country and BV status

Only species that were cultivable in the study are presented.

Lactobacillus species	Indian women		US women	
	Healthy	BV positive	Healthy	BV positive
L. acidophilus	1	–	–	–
L. coleohominis	–	–	1	2
L. crispatus	3	–	7	1
L. fermentum	1	–	–	–
L. gasseri	–	–	–	1
L. iners	–	–	2	–
L. jensenii	3	–	4	1
L. johnsonii	–	–	1	1
L. mucosae	1	–	–	–
L. reuteri	1	1	–	–
L. rhamnosus	–	–	2	–
L. ruminis	–	–	–	1
L. salivarius	1	–	–	–
L. vaginalis	–	–	1	–

from women with a Nugent score of 0–3, indicating normal vaginal microflora, and 23.2 % were obtained from women with a Nugent score of 7–10, indicating BV.

Seven *Lactobacillus* species were isolated from 11 of the 20 Indian women and nine species were isolated from 15 of the 20 US women. Of the 14 cultivable *Lactobacillus* species, seven (50 %) were from Indian women and nine (64 %) were from US women (Table 1). The predominant *Lactobacillus* species among Indian women were *L. crispatus*, *L. jensenii* and *L. reuteri*; while *L. crispatus*, *L. jensenii* and *L. coleohominis* were most commonly found among US women (Table 1).

Table 2. Distribution of cultivable *Lactobacillus* species isolates by BV status among US and Indian women

Percentages represent column totals.

	Healthy women, *n* (%)	BV-positive women, *n* (%)	Total, *n* (%)	*P* value
Any *Lactobacillus*				
Absent	86 (40.6)	122 (76.3)	208 (55.9)	**0.0001**
Present	126 (59.4)	38 (23.8)	164 (44.1)	
L. acidophilus				
Absent	124 (98.4)	38 (100)	162 (98.8)	1.000
Present	2 (1.6)	0 (0)	2 (1.2)	
L. coleohominis				
Absent	125 (99.2)	21 (55.3)	146 (89.0)	**<0.0001**
Present	1 (0.79)	17 (44.7)	18 (10.9)	
L. crispatus				
Absent	67 (53.2)	37 (97.4)	104 (63.4)	**<0.0001**
Present	59 (46.8)	1 (2.7)	60 (36.6)	
L. fermentum				
Absent	125 (99.2)	38 (100)	163 (99.4)	1.000
Present	1 (0.8)	0 (0)	1 (0.61)	
L. gasseri				
Absent	126 (100)	36 (94.7)	162 (98.8)	0.052
Present	0 (0)	2 (5.26)	2 (1.22)	
L. iners				
Absent	124 (98.4)	38 (100)	162 (98.8)	1.000
Present	2 (1.59)	0 (0)	2 (1.22)	
L. jensenii				
Absent	100 (79.4)	35 (92.1)	135 (82.3)	0.089
Present	26 (20.6)	3 (7.9)	29 (17.7)	
L. johnsonii				
Absent	122 (96.8)	35 (92.1)	157 (95.7)	0.354
Present	4 (3.2)	3 (7.9)	7 (4.3)	
L. mucosae				
Absent	121 (96.0)	38 (100)	159 (96.9)	0.590
Present	5 (3.97)	0 (0)	5 (3.05)	
L. reuteri				
Absent	116 (92.1)	28 (73.7)	144 (87.8)	**0.008**
Present	10 (7.94)	10 (26.3)	20 (12.2)	
L. rhamnosus				
Absent	124 (98.4)	38 (100)	162 (98.8)	1.000
Present	2 (1.59)	0 (0)	2 (1.22)	
L. ruminis				
Absent	126 (100)	36 (94.7)	162 (98.8)	0.052
Present	0 (0)	2 (5.26)	2 (1.22)	
L. salivarius				
Absent	119 (94.4)	38 (100)	157 (95.7)	0.202
Present	7 (5.56)	0 (0)	7 (4.27)	
L. vaginalis				
Absent	121 (96.0)	38 (100)	159 (96.9)	0.590
Present	5 (3.97)	0 (0)	5 (3.06)	

Table 3. Percentage of cultivable *Lactobacillus* species classified by glucose fermentation among women with and without BV in India and the USA

Glucose fermentation	BV positive, *n* (%)		BV negative, *n* (%)	
	India	USA	India	USA
Facultative heterofermentative	–	20 (71.4)	15 (26.8)	14 (20.6)
Obligate heterofermentative	10 (100.0)	–	16 (28.6)	5 (7.4)
Obligate homofermentative	–	8 (28.6)	25 (44.6)	49 (72.1)

Among the 26 colonized women, nine had more than one *Lactobacillus* species. *L. acidophilus*, *L. fermentum*, *L. iners*, *L. mucosae*, *L. rhamnosus*, *L. salivarius* and *L. vaginalis* were only isolated from women with normal microbiota, whereas the other predominant *Lactobacillus* species (*L. coleohominis*, *L. crispatus*, *L. jensenii*, *L. johnsonii* and *L. reuteri*) were isolated from women both with and without BV. Colonization by any lactobacilli, and in particular *L. crispatus*, was significantly associated with a normal vaginal microbiota, while *L. reuteri* and *L. coleohominis* were significantly associated with BV (Table 2). A majority of the *Lactobacillus* species among Indian women were heterofermentative (Table 3).

Our study shows that the vaginal flora of healthy Indian and US women is dominated by two cultivable *Lactobacillus* species (*L. crispatus* and *L. jensenii*), similar to the findings of Pavlova *et al.* (2002). These species have been suggested to be beneficial in maintaining a normal vaginal microflora and preventing sexually transmitted infections (Antonio *et al.*, 1999; Pavlova *et al.*, 2002; Antonio *et al.*, 2005; Damelin *et al.*, 2011). Our findings, however, differ from those of an Indian study by Garg *et al.* (2009), who found that *L. reuteri*, *L. fermentum* and *L. salivarius* were the main species in the vaginal flora of women in Delhi, northern India. These differences suggest that vaginal *Lactobacillus* species may vary in subpopulations of India.

Our findings reveal that despite differences in ethnicity and geographical setting, healthy women share similar vaginal *Lactobacillus* species (especially *L. crispatus* and *L. jensenii*), but that the vaginal ecology is altered when women develop BV. The finding that the vaginal microbiome was more heavily weighted toward heterofermentative species among the Indian women in our sample is consistent with the literature, but deserves more attention (Gonzalez *et al.*, 2011).

There are several limitations to this study. First, because we used culture, only species that could be cultured were identified. Selective media such as Rogosa or MRS agar do not support the growth of certain species such as *L. iners* (Fredricks *et al.*, 2005; Vasquez *et al.*, 2005). Cultivation-independent methods have found that *L. iners* is one of the most frequently isolated organisms from the vagina of healthy women (Vasquez *et al.*, 2002; Anukam *et al.*, 2006; Zhou *et al.*, 2007). Our negative result on isolation of *L. iners* with blood agar in samples from Indian women does not rule out the possibility that this species was not present in the population, because small colonies may have been easily overlooked. Secondly, the differences in the diversity and distribution of *Lactobacillus* species may have resulted from participants being in different phases of their menstrual cycle. Finally, findings from our study cannot be generalized to other populations because of the small sample size, non-probability sample and limited statistical power.

Despite these limitations, our study fills a gap in the literature since little is known about the vaginal flora of Indian women. These data suggest that more needs to be known about the microbial diversity of vaginal flora of women in different parts of the world and how it affects vaginal health. Larger studies using genotyping methods and longitudinal study designs are needed to better characterize this important vaginal defence.

ACKNOWLEDGEMENTS

For their generous assistance on this project, the authors would like to thank Seema Kotian, Keerthi Rao, Bhavya A. Manjunath, Rani Chinnappa and Fazila Begum at PHRII, without whose support this study could not have been carried out, and all of the women in the study for their participation. Special thanks to Dr Daniel Raiten at National Institutes of Health and Dr Chander Shekhar at the Indian Council of Medical Research for their continuing support and advice. This study was supported by the Indo-US Program on Maternal and Child Health and Human Development Research funded by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant 5R03HD055117-02) and the Indian Council of Medical Research (grant no. 63/1/ Indo-US/07-RHN). The study sponsors had no role in the study design, conduct, collection, management, analysis or interpretation of the data, or preparation, review or approval of the manuscript.

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