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Identification of culturable vaginal *Lactobacillus* species among reproductive age women in Mysore, India

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A healthy vaginal environment is predominated by certain *Lactobacillus* species, which lead to the prevention of infections of the reproductive tract. This study examined the characteristics of cultivable *Lactobacillus* species in both healthy women and women with bacterial vaginosis (BV). Between November 2011 and September 2013, 139 women attending a women's clinic in Mysore, India, were evaluated for BV in a cross-sectional study. BV was diagnosed using Amsel's criteria: homogeneous vaginal discharge, vaginal pH >4.5, production of amines, and presence of ''clue'' cells. Those with three or more of the characteristics were considered to have BV. Vaginal swabs were then cultured in Rogosa agar and de Man-Rogosa-Sharpe broth. Gram-positive lactobacilli generating 600–800 bp amplicons by16 sRNA were further characterized by sequencing. Cultivable vaginal samples were obtained from 132 women (94.9 %). According to the Amsel criteria, 83 women (62.1 %) were healthy, and 49 (37.1 %) had BV. Eleven different *Lactobacillus* species were isolated from 47 women. The common lactobacilli species found in this sample included *L. crispatus* (39.6 %), *L. gasseri* (45.8 %), and *L. jensenii* (14.6 %). Lactobacilli were isolated from 39 healthy women and eight with BV. *L. gasseri* was cultured from 18.8 % of healthy women and 6.1 % with BV. The presence of *L. reuteri* was significantly associated with normal vaginal microbiota (*P*-value=0.026). These results further our understanding of vaginal lactobacilli colonization and richness in this particular population. Our findings showed that lactobacilli species present in the vaginas of healthy women in India do not differ from those reported from other countries.

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INTRODUCTION

A growing body of research suggests that the vaginal microbiome has evolved to protect women against a variety of pathogens including parasitic, bacterial and viral infections (Nardis *et al.*, 2013; Razzak *et al.*, 2011; Rendón-Maldonado *et al.*, 1998). Genus *Lactobacillus*, Gram-positive bacteria first identified by Döderlein in 1928, are the best studied of the facultative microbes found in the vaginal environment (van de Wijgert *et al.*, 2014). Research using culture-based methods among women living mostly in industrialized countries first suggested that a healthy vaginal flora was dominated by four species: *L. fermentum; L. brevis; L. jensenii*; and *L. casei* (Antonio *et al.*, 1999). More recently, studies using molecular methods in similar populations have narrowed the predominant species seen in the vaginal mucosa of most reproductive age women to *L. crispatus, L. iners* and *L. gasseri* (Linhares *et al.*, 2010).

There are only a few studies characterizing the vaginal *Lactobacillus* species found among healthy women of

Abbreviations: BV, bacterial vaginosis; RTI, reproductive tract infection

reproductive age in India. A study among 80 women of reproductive age carried out by Garg *et al.* (2009) found the predominant species isolated were *L. reuteri*, present in 32.5 % (n=26), *L. fermentum* in 25 % (n=20), and *L. salivarius* in 16.3 % (n=13) of women (Garg *et al.*, 2009). They suggested that this may mean that the species found in the vaginal microbiome of healthy Indian women may vary widely from those found in women from other countries (Antonio *et al.*, 1999). Findings from a pilot study carried out by our group using 16 sRNA in vaginal samples obtained from 40 healthy women of reproductive age (20 from each country) from Mysore, India, and San Francisco, USA, showed instead that the vaginal *Lactobacillus* species in both groups were similar, with *L. jensenii* and *L. crispatus* dominating all other species (Madhivanan *et al.*, 2014).

Growing evidence also shows that bacterial vaginosis (BV) leads to changes in the vaginal microbiome (Ma *et al.*, 2012). BV has been shown to cause the loss of *L. crispatus* species, and has been associated with a more complex ecology of bacterial communities. Women with BV also have fewer vaginal *Lactobacillus* species, with the most common being *L. iners*, BVAB2, *L. crispatus, L. jensenii, L. reuteri* and *L. coleohominis* (Madhivanan *et al.*, 2014; Tamrakar *et al.*, 2007). Interestingly, our group found that when Indian women were compared with their counterparts in the USA, the *Lactobacillus* species found were more commonly obligatively heterofermentative, suggesting a need for more metabolic flexibility and a natural selection process that favoured species protective against a larger number of pathogenic threats (Madhivanan *et al.*, 2014). The present study characterized the cultivatable species of *Lactobacillus* in a large sample of women of reproductive age with and without BV in Mysore, India.

METHODS

Study population. Between November 2011 and September 2013, potential participants were recruited from a consecutive sample of women attending the reproductive health clinic in Mysore, India. This cross-sectional study characterized the vaginal *Lactobacillus* species in women with and without BV. Detailed methods are described elsewhere (Madhivanan *et al.*, 2014). In brief, women were assessed for eligibility based on a series of questions. To be included in the study, participants had to be between the ages of 18 and 45 years, sexually active (defined as having had vaginal intercourse at least once in the three months prior to enrolment) and having the ability to provide informed consent. Women who were pregnant, having menses or a diagnosis of any reproductive tract infection (except BV) were excluded from the study. In addition, women who had used any antibiotics in the prior 30 days were also excluded. All eligible women were provided details about the study, and the research staff answered any questions they had before obtaining informed consent from each participant in Kannada, the language spoken by the majority of local residents.

Human subjects. The study was reviewed and approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley; Florida International University and the Public Health Research Institute of India's Institutional Review Board, and conducted in compliance with all federal regulations governing the protection of human subjects. Eligible women who gave written informed consent were assigned a study identification number (SID). All subsequent documents were coded with the SID and had no personal identifiers.

Clinical evaluation for bacterial vaginosis. A trained physician conducted physical examinations of all study participants and collected biological specimens for detection of reproductive tract infections (RTIs). The diagnosis of BV was based on the Amsel criteria of having any three of the four following characteristics: a vaginal pH $>$4.5, the presence of a fishy amine odour upon addition of 10 % potassium hydroxide (KOH), a homogeneous white vaginal discharge and the presence of clue cells on wet-mount microscopy (Amsel *et al.*, 1983). Three vaginal specimens were obtained by placing a cotton swab in the posterior fornix of the vagina. The first swab was used to measure the vaginal pH, smeared onto two microscope slides and then placed in a tube containing four drops of normal saline to prepare a slide for wet-mount microscopic examination. The remaining two swabs were placed into BBL Port-A-Cul tubes (Becton, Dickinson) and stored in a refrigerator until they were transported to the laboratory for further processing. Microscope slides were air-dried, fixed with methanol, and subsequently Gram-stained for Nugent scoring (Nugent *et al.*, 1991).

The vaginal samples were classified according to Nugent scoring of Gram-stained vaginal smears, which is a standardized 0–10 point scoring system based on the presence of three bacterial morphotypes: large Gram-positive rods (*Lactobacillus* spp.), small Gram-negative or Gram-variable coccobacilli (*Gardnerella* and anaerobic spp.), and curved Gram-variable rods (*Mobiluncus* spp.) A score of 0–3 was considered normal, 4–6 was intermediate, and scores $\geq$7 indicated BV. The healthy women according to Nugent score had none or no more than one of the four characteristics based on Amsel criteria, and none of them had clue cells on wet-mount microscopy. Two study personnel carried out the Nugent scoring independently, and the inter-observer agreement was excellent (kappa=0.84). All discordant slides (n=8) were read by a third reader as a tiebreaker. This study included women with normal or BV Nugent scores.

Laboratory methods. Laboratory tests were performed by trained research assistants using standardized protocols in the Public Health Research Institute of India (PHRII) laboratory located 10 min driving distance from the clinic. The two vaginal swabs that were stored and transported in Port-A-Cul tubes (Becton Dickinson) from the clinic to the laboratory were then plated on Rogosa and sheep blood agar (HiMedia Laboratories) within 12 h of sample collection. Plates were incubated for 24 to 48 h at 37 °C in 5 % CO_2 anaerobic jars containing AnaeroPacks (Mitsubishi). Only plates containing 10–200 c.f.u. were analysed further. Up to 10 colonies from each plate were randomly selected for processing. The colonies with Gram-positive rods were inoculated in de Man-Rogosa-Sharpe (MRS) broth medium. Individual colonies were cultured in 2.5 ml MRS broth (BD Diagnostics), incubated overnight at 37 °C in 5 % CO_2. Glycerol (10 %) stocks were prepared and stored in 200 µl of the broth culture at −80 °C. The remainder was used for DNA extraction.

Bacterial DNA extraction. DNA was extracted from the broth cultures for 16S rDNA PCR and sequence analysis. A 1 mL aliquot of the culture was centrifuged for 5 min at 5000 r.p.m. The pellet was resuspended in 500 ml of autoclaved double-distilled water in a sterile cryotube containing 0.5 mm glass beads (Research Products International). Samples were vortexed, and a freeze–thaw method was used for DNA extraction. They were frozen overnight at −20 °C, then thawed and vortexed again. Finally, they were centrifuged for 30 s at 5000 r.p.m. before PCR amplification.

PCR amplification of 16S rDNA sequences. PCR amplification was carried out with 5.2 µl of DNA template in 30 µl of PCR mixture. Each reaction mixture contained 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 U of *Taq* polymerase (Genei), 1 × *Taq* reaction buffer, and 1 µM of primers. The 16S rDNA PCR assays were carried out with the primers 16s8F (AGAGTTTGATCCTGGCTCAG) and 16s806R18 (GGACTACCAGGGTATCTAATCC), as described previously (Martinez-Freijo *et al.*, 1998). The 800 bp PCR product was obtained under the following conditions: 94 °C for 5 min, 30–35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 90 s. The PCR products were electrophoresed on 1 % agarose gels and stained with ethidium bromide and visualized under UV transillumination. *Escherichia coli* ATCC 25922 DNA extracted by the freeze–thaw method was used as a positive control for the 16S rDNA.

Sequencing analysis of 16S rDNA. PCR products were purified by AxiPrep kit (Axigen) or Genejet gel extraction kits (Fermentas). Sequencing was done at SciGenome or Eurofins Genomics India (Bangalore). DNA sequences ranging between 400 and 600 bp or more for the 16S rDNA gene were visually inspected, aligned and compared against sequences deposited in GenBank by BLAST (National Center for Biotechnology Information) or the Greengenes server with the Align tool (*greengenes.lbl.gov*). 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 entered in an Access database (Microsoft Corporation). Conventional descriptive statistics were used to assess the distribution of *Lactobacillus* species among the study participants. The Fisher exact test was used to test the relationship between BV status (normal microbiota versus BV) and the presence of any lactobacilli and specific *Lactobacillus* species. The Pearson chi-squared test was used to examine the association of sociodemographic variables and BV status. Two-tailed *P*-values <0.05 were considered statistically significant. Only samples with confirmed BLAST identity ≥ 95 %, and match length ≥ 500 base pairs were included in the analyses. About 2644 of the 2680 samples had colonies that met the requirements of BLAST identity ≥ 95 % and match length ≥ 500 base pairs. Data were analysed with SAS version 9.3 (SAS Institute).

RESULTS

Of the 183 women screened, vaginal samples were obtained from 139, and 132 were included in the analyses, as the remaining seven did not have complete information. Eighty-three women had normal microbiota and 49 (37.1 %) were classified as BV by Nugent score. The primary reason for participants to attend the reproductive health clinic was a routine health check-up that was provided at this clinic on a regular basis at no cost. Demographic characteristics are shown in Table 1 for the two most common species of *Lactobacillus* present in this study population. The majority of the women were married and had a single sex partner. About 87 % of the participants reported their religion as Hindu. None of the women reported any douching practices. While 81 % of the study participants had undergone tubal ligation as the primary family planning method, about 11.9 % reported not using any contraception methods. None of the characteristics including contraceptive methods had any association with BV status. Women with *L. crispatus or L. gasseri* were significantly older and had more than a primary school education.

Table 1. Sociodemographic characteristics and prevalence of *L. crispatus* and *L. gasseri* in 132 women in Mysore, India (November 2011 to September 2013)

	L. crispatus		*L. gasseri*		
Characteristic	***N***	**%**	***N***	**%**	***P*-value**
Age (years)					0.042
20–29	58	43.9	9	27.3	
30–39	67	50.8	23	69.7	
40–49	7	5.3	1	3.0	
Education (years)					0.016
0	26	19.7	2	6.1	
1–7	55	41.7	12	36.4	
8–12	49	37.1	19	57.6	
>12	2	1.5	0	0.0	
Religion					0.560
Hindu	115	87.1	30	90.9	
Other	17	12.9	3	9.1	
Years married to current partner					0.119
0–11	57	43.2	11	33.3	
12–24	69	52.3	22	66.7	
25–37	6	4.5	0	0	
Method of preventing pregnancy					
None	16	11.9	1	3.0	0.118
Condoms	4	3.0	2	6.1	0.254
IUD	1	0.7	1	3.0	0.246
Pill	1	0.7	0	0.0	1.000
Withdrawal	2	1.5	2	6.1	0.059
Tubal ligation	109	81.3	27	81.8	0.936
BV*					0.008
Positive	49	37.4	6	18.2	
Negative	82	62.6	27	81.8	

BV, Bacterial vaginosis.
*Nugent score was missing for one participant.

Lactobacillus colonies were recovered from 47 (35.6 %) of the 132 women. While 32 (24.2 %) of the 132 women did not have any bacterial growth culturable by either Rogosa or MRS medium, 45 women did show growth, but it was not *Lactobacillus*. These women were demographically no different than the women who did have *Lactobacillus* colonies. Another eight women (6.0 %) had growth that produced no bands by aragose gel electrophoresis. Based on the Nugent score of vaginal smears, lactobacilli were isolated from 39/83 (46.9 %) women with normal microbiota and 8/49 (16.3 %) women with BV. The predominant *Lactobacillus* species cultured in this sample included *L. crispatus* (40.4 %) and *L. gasseri* (44.7 %).

Table 2 shows the distribution of colonizing *Lactobacillus* stratified by BV status in the sample. More than one species of *Lactobacillus* was cultured from 22 (46.8 %) of the 47 women who had *Lactobacillus* identified from the vaginal samples. About eight (17.0 %) women had at least three or

Table 2. Bacterial vaginosis (BV) status and distribution of colonizing *Lactobacillus* species in 132 women in Mysore, India (November 2011 to September 2013)

Colonizing species	Women colonized (*n*)	BV status	
		Healthy (*n*)	BV+(*n*)
L. acidophilus	0	–	–
L. coleohominis	4	4	–
L. crispatus	19	16	3
L. fermentum	6	6	–
L. gasseri	21	18	3
L. iners	1	1	–
L. jensenii	7	7	–
L. johnsonii	4	4	–
L. mucosae	2	2	–
L. oris	2	2	–
L. reuteri	9	9	–
L. rhamnosus	0	–	–
L. ruminis	2	–	2
L. salivarius	0	–	–
L. thermophilus	2	2	–
L. vaginalis	0	–	–

Only species that were cultivable are included in the table.

Table 3. Bacterial vaginosis (BV) status and distribution of cultivable *Lactobacillus* species isolates in 132 Indian women in Mysore, India (November 2011 to September 2013)

	Healthy		BV+		Total		
Species	*n* (%)		*n* (%)		*N* (%)		*P*-value*
Any *Lactobacillus*							
Absent	44	(53.0)	41	(83.7)	85	(63.9)	0.0004
Present	39	(47.0)	8	(16.3)	47	(35.6)	
L. coleohominis							
Absent	79	(95.2)	49	(37.7)	128	(97.0)	0.296
Present	4	(4.8)	0	(0.0)	4	(3.0)	
L. crispatus							
Absent	67	(80.7)	46	(93.9)	113	(85.6)	0.036
Present	16	(19.3)	3	(6.1)	19	(14.4)	
L. fermentum							
Absent	77	(92.8)	49	(100)	126	(95.5)	0.086
Present	6	(7.1)	0	(0)	6	(4.5)	
L. gasseri							
Absent	65	(77.4)	46	(93.9)	111	(83.5)	0.015
Present	18	(22.6)	3	(6.1)	21	(16.5)	
L. iners							
Absent	82	(98.8)	49	(100)	131	(99.2)	1.000
Present	1	(1.2)	0	(0)	1	(0.8)	
L. jensenii							
Absent	76	(91.6)	49	(100.0)	125	(94.7)	0.047
Present	7	(8.3)	0	(0.0)	7	(5.3)	
L. johnsonii							
Absent	79	(95.2)	49	(100.0)	128	(97.0)	0.296
Present	4	(4.8)	0	(0.0)	4	(3.0)	
L. mucosae							
Absent	81	(97.6)	49	(100.0)	130	(98.5)	0.530
Present	2	(2.4)	0	(0.0)	2	(1.5)	
L. oris							
Absent	81	(97.6)	49	(100.0)	130	(98.5)	0.533
Present	2	(2.4)	0	(0)	2	(1.5)	
L. reuteri							
Absent	74	(89.2)	49	(100.0)	124	(93.2)	0.026
Present	9	(10.8)	0	(0.0)	9	(6.8)	
L. ruminis							
Absent	81	(100)	47	(95.9)	130	(98.5)	0.134
Present	0	(0)	2	(4.1)	2	(1.5)	
L. thermophilus							
Absent	81	(97.6)	49	(100)	130	(98.5)	0.530
Present	2	(2.4)	0	(0)	2	(1.5)	

**P*-values obtained by chi-square tests when expected cell frequency was <5, otherwise by Fisher's exact tests.

more different *Lactobacillus* species simultaneously detected in their vaginal flora. *L. coleohominis*, *L. fermentum*, *L. iners, L. johnsonii*, *L. jensenii*, *L. mucosae, L. oris*, *L. reuteri* and *L. thermophilus* were only isolated from women with normal microbiota whereas the other predominant *Lactobacillus* species (*L. crispatus*, *L. gasseri*) were isolated from both women with and without BV, and *L. ruminis* was isolated only from women with BV. Women with normal microbiota had at least one of 11 *Lactobacillus* species, while women with BV had only three *Lactobacillus* species present: *L. crispatus, L. gasseri and L. ruminis. The Lactobacillus* species that we looked for, but were not recovered in this study sample, included *L. acidophilus, L. brevis, L. buchneri, L. casei, L. catenaformis, L. confusus, L. parabuchneri, L. rhamnosus,* and *L. salivarius*. Colonization by any *Lactobacillus* (P=0.0003) and in particular *L. crispatus* (P=0.042), *L. jensenii* (P=0.047), *L. gasseri* (P=0.015), or *L. reuteri* (P=0.026) was significantly associated with a normal vaginal microbiota (Table 3).

DISCUSSION

Our study shows the vaginal flora of Indian women was dominated by two cultivable *Lactobacillus* species (*L. crispatus* and *L. gasseri*). Research from different parts of the world shows that *L. crispatus, L. gasseri* and *L. jensenii* are the most common cultivable *Lactobacillus* species, and this was true in our study sample as well (Chaban *et al.*, 2014; Mendes-Soares *et al.*, 2014; van de Wijgert *et al.*, 2014). Unlike other research findings, we found frequent co-occurrence of species as opposed to dominance of one species or another. Vaginal colonization predominantly with *L. gasseri, L. crispatus, L. reuteri* and *L. jensenii* in our sample shows a distribution similar to that found in women in other regions of the world (Antonio *et al.*, 1999; Damelin *et al.*, 2011; Dong-hui *et al.*, 2009; Song *et al.*, 2000; Vásquez *et al.*, 2002). While *L. crispatus* and *L. gasseri* were detected in women with and without BV, they were

found in a greater number of women with normal microbiota than in women with BV. The strong association of *L. crispatus* with normal vaginal microbiota is similar to that found in several other studies (Antonio *et al.*, 1999; Vásquez *et al.*, 2002). In a longitudinal study of vaginal microbiota, Verstraelen *et al.* (2009) showed that *L. crispatus* was associated with the stability of the vaginal flora. These findings however, differ greatly from 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* may vary in subpopulations of India.

There are several study limitations that should be considered when interpreting the findings. First, as our methodology used culture first to detect *Lactobacillus* species, only species that can be cultured were further identified. Our study only provides a limited view of the species of *Lactobacillus* that may be present in the vaginal environment. Second, the differences found in the diversity and distribution of the *lactobacillus* species may have resulted from participants being in different phases of their menstrual cycle. While the relationship of hormones to the endogenous vaginal flora is important, it cannot be ascertained from our study. Our negative result for isolation of *L. iners* does not mean that this species may not be present in the population, as we may have been limited in using a selective media that was not supportive for the growth of this species. Finally, findings from our study cannot be generalized to other populations due to the small sample size, non-probability sample coming from a reproductive health clinic and limited statistical power.

Our study has several strengths as well. Since little is known about the vaginal flora of Indian women, our study contributes to the literature and knowledge base by providing the characteristics of cultivable lactobacillus present among south Indian women. The findings of this study also confirm the prominence and wide carriage of several *Lactobacillus* species, suggesting that they may be of particular importance in the development of future microbicides and probiotics. The vaginal species of *Lactobacillus* found in south Indian women from Mysore are similar to those identified in women from other populations. Our findings need to be confirmed in a larger study using molecular methods and longitudinal study designs to better characterize this important vaginal defence.

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