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Spectrum of *Lactobacillus* species present in healthy vagina of Indian women

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***Background & objectives*: Lactobacilli are depleted in vagina of women suffering from recurring episodes of bacterial vaginosis with vaginal *p*H ≥5. With the objective of making available probiotic lactobacilli for replenishment in such women, a study was undertaken to isolate and characterize the Lactobacilli present in women with eco-healthy vagina in Delhi. No information is so far available on the species of Lactobacilli resident in vagina of women in India.**

***Methods*: Vaginal swabs were taken from 80 women with informed consent after ethical approval and grown in MRS broth. Gram-positive, catalase-negative bacilli generating about 200 bp amplicon by PCR with *Lactobacillus* genus specific primers were further characterized by employing species specific primers followed by sequencing of 16S rDNA. Isolates of the same species were differentiated by random amplified polymorphic DNA (RAPD) profiles.**

***Results*: The predominant species isolated were *L. reuteri* present in 26 (32.5%) women, *L. fermentum* in 20 (25%), and *L. salivarius* in 13 (16.25%) women. Sequencing of 16S rDNA of 20 isolates showed that except for two isolates of *L. plantarum*, sequences of the remaining agreed well with PCR identification. None of the isolates had similar RAPD profile.**

***Interpretation & conclusions*: Our findings showed lactobacilli species present in healthy vagina of women in India differ from those reported from other countries. This information would be useful to development of probiotic tablets seeking to replenish the missing lactobacilli for reproductive health of women in India.**

Key words Bacterial vaginosis - *Lactobacillus* - PCR - RAPD - 16S rDNA sequencing - variable strains

The role of probiotics in conferring benefit to the host is being increasingly realized. However, most reports are on probiotics in dairy products which are of industrial importance and employed in fermented food items. Few reports are available on probiotics resident in female reproductive tract even though the presence of lactobacilli in vagina was first observed by Doderlein in 1894[1]. By virtue of their non pathogenic character, their ability to make lactic acid and thereby keeping vaginal *p*H around 4[2], secrete H_2O_2[3], and bacteriocins[4], lactobacilli presumably exercise local anti-microbial action against pathogens.

During the course of a study in women with recurring episodes of bacterial vaginosis (BV), caused

by a variety of reproductive tract infections (RTIs), it was noted that the vaginal *p*H of many women was between 5-7 and that no lactobacilli could be isolated from majority of these women (unpublished observations). Treatment with antibiotics and drugs brought in relief only temporarily. They required replenishment of probiotic lactobacilli for hopefully better reproductive health. As no information was available on the species of lactobacilli inhabiting vagina of women in India, this study was undertaken to isolate and identify lactobacilli resident in healthy vagina of women in Delhi.

Material & Methods

Sampling: With approval of the Institutional Ethics Committee of Sir Ganga Ram Hospital, New Delhi, and after obtaining informed consent of the subjects; vaginal isolates were collected during 2 years from January 2004 to January 2006 from 80 women visiting OPD for either family planning contraceptives or antenatal and post-natal care. The subjects examined were of reproductive age (18-45 yr, mean 26 $\pm$ 3 yr) with healthy vagina having *p*H 4-4.5, Nugent score $\leq$ 4, and no visible infection as seen by speculum examination. A sterile swab was rolled over high vaginal wall and placed in sterile screw cap tubes containing MRS (deMan, Rogosa and Sharpe) broth (Hi-Media, India). After bringing the samples to the Talwar Research Foundation Laboratory, New Delhi, the swab was spread on BCP-MRS agar plate. BCP (Bromo-cresol purple, Merck, India) is a colour indicator dye with *p*H range 5.2-6.8. It turns from purple to yellow with lowering of *p*H, thus the colony producing lactic acid is identifiable by the yellow color. The plate was incubated at 37°C in anaerobic jar for 24-48 h. A representative single colony was selected from each isolate and Gram stained. Catalase test was done by pouring a drop of H_2O_2 on a colony, and absence of oxygen bubble formation indicated absence of catalase[5]. Gram-positive, catalase negative colonies were cultured individually in MRS broth and stored in 20 per cent glycerol at -20°C.

Genus and species identification by PCR: DNA was isolated by the method described by Pospiech & Neumann[6] with minor modifications. Two ml of culture in MRS broth was centrifuged at 6,000 *g* for 10 min, and the pellet obtained was suspended in SET buffer (NaCl 75 mM, EDTA 25 mM, Tris 20 mM, *p*H 7.5) containing 1mg/ml lysozyme (Amresco, USA) and incubated at 37°C for 1 h. Cell debris was removed by lysis with 10 per cent sodium dodecyl sulphate (SDS) for 30 min at 37°C and precipitation by 5M NaCl for 30 min. DNA was extracted with chloroform: isoamylalcohol (24:1), precipitated with isopropanol at -20°C, washed with 70 per cent ethanol and dried under vacuum. The DNA was then suspended in 20 µl of TE buffer (Tris 10 mM, EDTA 1 mM, *p*H 8.0). Each isolate was then identified to the genus level by amplification with genus specific primer LbLMA-rev (5' CTC AAA ACT AAA CAA AGT TTC 3') and a universal primer R16-1 (5' CTT GTA CAC ACC GCC CGT TCA 3') using programme as initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C and extension at 72°C for 30 sec and 7 min thereafter[7]. After confirming the genus, multiplex PCR-G was employed to determine the group to which an isolate belonged to using primer mix containing equimolar 4 forward primers namely Ldel-7(5'ACAGATGGATGGAGAGCAGA 3'), LU-1'(5'ATTGTAGAGCGACCGAGAAG3'), LU-3'(5'AAACCGAGAACACCGCGTT 3'), LU-5' (5'CTAGCGGGTGCACTTTGTT3')and 1 common reverse primer Lac-2 (5'CCTCTTCGCTCGCCGCTACT 3') as per Song *et al*[8]. The primers were got synthesized by order from Sigma, Bangalore. PCR programme (PCR-G) employed initial denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 20 sec, annealing and extension at 55°C for 2 min; and final extension at 74°C for 5 min. Species were identified by multiplex PCR assays as given in Table I. PCR programme and reaction mix was same as for PCR-G except annealing temperature which was 68°C for PCR II-1, 65°C for PCR II-2, 62°C for multiplex PCR III and 60°C for PCR IV. Amplicons were analysed by electrophoresis in 2 per cent agarose gel followed by ethidium bromide staining.

RAPD analysis: Random amplified polymorphic DNA (RAPD) analysis was done to differentiate various strains of the same species isolated. The primer used was 5' AGT CAG CCA C 3' (Sigma, USA) as per Tynkkynen *et al*[9]. The reaction mix contained 30 ng of template DNA in PCR buffer with 2 mM $MgCl_2$, 0.2 mM of each nucleotide and 2.5U of Taq polymerase (Life Technologies, India) in a total volume of 25µl. PCR amplification was conducted in an Applied Biosystems Thermal Cycler 2400 (USA) with the following temperature profiles, initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 sec, 32°C for 2 min, 72°C for 2 min, and final extension at 72°C for 5 min. PCR products were visualized on 1.5 per cent agarose gel. Each strain of a given species having different RAPD pattern was numbered separately.

Table I. Species identified by multiplex PCR

Programme	Primer (5'-3' sequence)	Species	Amplicon
PCR II-1	L-aci-1 (TGCAAAGT GGTAGCGTAAGC)	*L. acidophilus*	210 bp
	23-10C		
	CCTTTCCCTCACGGTACTG		
	Ljen-3	*L. jensenii*	700 bp
	AAGAAGGCACTGAGTACGGA		
	23-10C		
PCR II-2	Lcri-3	*L. crispatus*	522 bp
	AGGATATGGAGAGCAGGAAT		
	Lcri-2		
	CAACTATCTCTCTTACACTGCC		
	Lgas-3	*L. gasseri*	360 bp
	AGCGACCGAGAAGAGAGAGA		
	Lgas-2		
	TGCTATCGCTTCAAGTGCTT		
PCR-III	LU-5	*L. paracasei*	312 bp
	Lpar-4		
	GGCCAGCTATGTATTCACTGA		
	LU-5	*L. rhamnosus*	113 bp
	RhaII		
	GCGATGCGAATTTCTATTATT		
	Lsal-1	*L. salivarius*	411 bp
	AATCGCTAAACTCATAACCT		
	Lsal-2		
	CACTCTCTTTGGCTAATCTT		
	Lreu-1	*L. reuteri*	303 bp
	CAGACAATCTTTGATTGTTTAG		
PCR-IV	Lreu-4		
	GCTTGTTGGTTTGGGCTCTTC		
	Lpla-3	*L. plantarum*	248 bp
	ATTCATAGTCTAGTTGGAGGT		
	Lpla-2		
	CCTGAACTGAGAGAATTTGA		
	Lfer-3	*L. fermentum*	192 bp
	ACTAACTTGACTGATCTACGA		
	Lfer-4		
	TTCACTGCTCAAGTAATCATC		

16S rDNA sequencing: Sequencing of 16S rDNA of selected strains of each species of *Lactobacillus* was carried out at the National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra. PCR was done using 0.1 mM of primer-forward : 5' AGA GTT TGA TCC TGG CTC AG 3' reverse; 5' CCC ACT GCT GCC TCC CGT AG 3', 200 ng of template DNA, 0.2 mM of dNTPs, and 2.5U Taq polymerase[10]. The PCR programme was: initial denaturation at 93^{0}C for 3 min, followed by 30 cycles at 93^{0}C for 1 min, 48^{0}C for 1 min, 72^{0}C for 2 min and final extension at 72^{0}C for 10 min. PCR mix was run on 1.5 per cent agarose gel at 120 V and 350 bp DNA fragment was eluted from the gel with QIAEX II gel extraction kit (Qiagens, Germany). The eluted DNA fragment was again amplified by PCR in a reaction mixture containing 8 µl of termination ready reaction mix (ABI PRISM, USA), 200 ng of PCR product DNA, 3.2 pmol of one Edwards primer, in a total volume of 20 µl. 35 cycles of sequencing PCR at 94^{0}C for 10 sec, 48^{0}C for 5 sec, 60^{0}C for 4 min was performed, PCR product was purified by adding 0.1 vol. of 3 M sodium acetate (*p*H 4.5) and 2.5 vol. of absolute ethanol. The sequencing of the amplicon was carried out using the ABI PRISM 310 genetic analyser. The sequence generated by the programme was identified through BLAST search[11].

Results & Discussion

Vaginal isolates from 80 women grown on MRS medium and characterized as *Lactobacillus* on the

basis of Gram positivity and catalase negativity were all observed to give an amplicon of about 200 bp by genus specific PCR (Fig. 1). Employing Group specific PCR it was observed that 80 per cent of the isolates belonged to Group IV, Group II was present in 13.75 per cent, and Group III in 6.25 per cent of women. No isolate belonged to Group I (Table II).

These 80 isolates were then analysed to determine the species by species specific primers. The most frequently encountered species were *L. reuteri* in 26 (32.5%) women, followed by *L. fermentum* 20 (25%), *L. salivarius* 13 (16.25%) and *L. plantarum* in 5 (6.25%) women. These 4 *Lactobacillus* species interestingly all belong to Group IV. Other species isolated were *L. crispatus* and *L. rhamnosus* each in 4 (5%) women, *L. jensenii* in 3 (3.75%), *L. gasseri* and *L. acidophilus* each 2 (2.5%) and *L. paracasei* in 1(1.25%) women. None of the isolate from the 80 women was *L. casei* or *L. delbruckii* (Table II). Fig. 2 shows the PCR products of 2 isolates each of the 6 species and 1 isolate each of the rest of 4 species isolated from 80 women.

Sequencing of 16S rDNA of a few isolates of each species (total n=20) was done to confirm the species as indicated by species specific PCR. Except for 2 isolates which were classified as *L. plantarum* but were identified as *L. pentosus* by sequencing, the sequence of the remaining isolates analysed agreed with the PCR identification (Table III).

To enquire whether isolates of the same species in a number of women were identical or different; their RAPD profiles were compared. It was noted that none of the isolates amongst 80 had similar profile (Fig. 3).

Reports on the species of Lactobacilli resident in healthy vagina of women from many countries are available. Vasquez *et al*[12] reported presence of *L. crispatus* in 47.8 per cent, *L. gasseri* in 30.4 per cent and *L. jensenii* in 17.4 per cent of 23 Swedish women examined using the same primers used in this study. Vallor *et al*[13] reported the predominant species as *L. jensenii* (41%) and *L. crispatus*

Table II. *Lactobacillus* species isolated from healthy vagina of women (n=80)

Group	Species	Number of isolates identified (%)
Group IV: 64 (80%)	*L. reuteri*	26 (32.5%)
	L. fermentum	20 (25%)
	L. salivarius	13 (16.25%)
	L. plantarum	5 (6.25%)
Group II: 11 (13.75%)	*L. crispatus*	4 (5%)
	L. jensenii	3 (3.75%)
	L. gasseri	2 (2.5%)
	L. acidophilus	2 (2.5%)
Group III: 5 (6.25%)	*L. casei*	0 (0)
	L. paracasei	1 (1.25%)
	L. rhamnosus	4 (5%)
Group I: 0	*L. delbruckii*	0 (0)

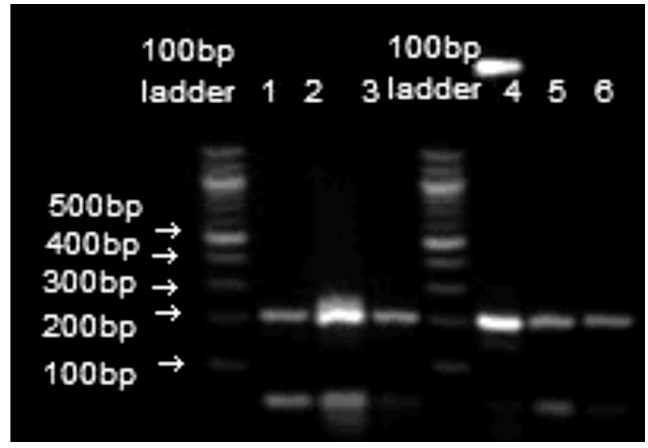

Fig. 1. Representative genus specific PCR products of 6 isolates (lanes 1-3, 4-6) on 2 per cent agarose gel. All 80 isolates gave a 200 bp amplicon.

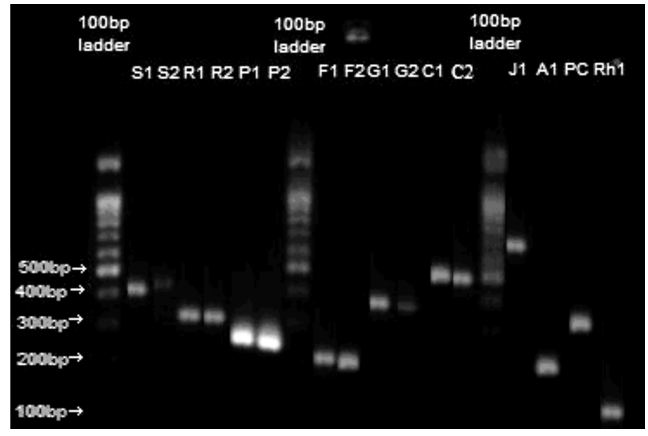

Fig. 2. Species-specific PCR products of the 10 species identified amongst 80 genus specific isolates of Lactobacilli. The representative figure gives the profiles of 2 isolates of 6 species and 1 isolate of the rest of 4 species viewed on 2 per cent agarose gel. S1 and S2, *L. salivarius*, 400 bp product with primers Lsal-1 and Lsal-2. R1 and R2, *L. reuteri*, 300 bp product with primers Lreu-1 and Lreu-4. P1 and P2, *L. plantarum*, 250 bp amplicon with primers Lpla-3 and Lpla-2. F1 and F2, *L. fermentum*, 200bp amplicon with primers Lfer-3 and Lfer-4. G1 and G2, *L. gasseri*, 350bp product with primers Lgas-1 and Lgas-2. C1 and C2, *L. crispatus*, 500bp PCR product with primers Lcri-1 and Lcri-2. J1, *L. jensenii*, 700bp amplicon with primers Ljen-3 and 23-10C. A1, *L. acidophilus*, 200bp PCR product with primers Laci-1and 23-10C. PC, *L. paracasei*, 300bp product with primers LU-5 and Lpar-4. Rh1, *L. rhamnosus*, 100bp product with primers LU-5 and RhaII.

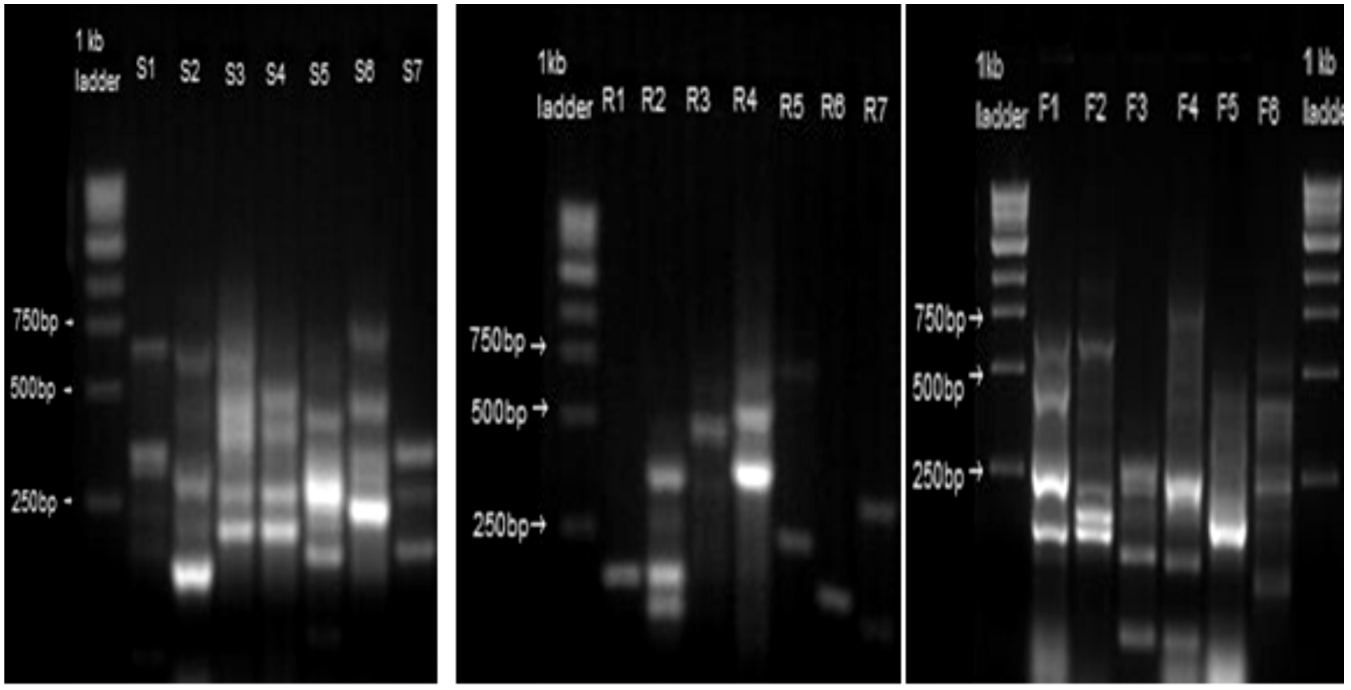

Fig. 3. RAPD profiles of selected strains of the predominant species isolated from different women. A. S1 to S7, *L. salivarius* isolates. B. R1 to R7, *L. reuteri* isolates. C. F1 to F6, *L. fermentum* isolates.

Table III. Species identification by two different genotypic approaches

Isolate no.	RAPD type	Species designation by	
		Species specific PCR	Sequencing of 16S rDNA
LB-04	S1	*L. salivarius*	*L. salivarius*
LB-09	S2	*L. salivarius*	*L. salivarius*
LB-30	S3	*L. salivarius*	*L. salivarius*
LB-45	S4	*L. salivarius*	*L. salivarius*
LB-36	F1	*L. fermentum*	*L. fermentum*
LB-39	F2	*L. fermentum*	*L. fermentum*
LB-60	F3	*L. fermentum*	*L. fermentum*
LB-65	F4	*L. fermentum*	*L. fermentum*
LB-73	F5	*L. fermentum*	*L. fermentum*
LB-01	R1	*L. reuteri*	*L. reuteri*
LB-23	R2	*L. reuteri*	*L. reuteri*
LB-55	R3	*L. reuteri*	*L. reuteri*
LB-61	P1	*L. plantarum*	*L. plantarum*
LB-75	P2	*L. plantarum*	*L. pentosus*
LB-80	P3	*L. plantarum*	*L. pentosus*
LB-10	C1	*L. crispatus*	*L. crispatus*
LB-08	G1	*L. gasseri*	*L.gasseri*
LB-42	J1	*L .jensenii*	*L. jensenii*
LB-12	A1	*L. acidophilus*	*L. acidophilus*
LB-13	Rh1	*L. rhamnosus*	*L. rhamnosus*

(38%) in USA. Burton *et al*[14] from Canada reported that of the 14 subjects harbouring Lactobacilli, *L. iners* was present in 8 (57.1%) and *L. crispatus* in 7 (50%) women, these two species co-existed in 2 subjects. These two species of lactobacilli were relatively rare in their occurrence in India. On the other hand, the more frequent lactobacilli present were *L. reuteri* and *L. fermentum*. These along with *L. salivarius* and *L. plantarum* were present in 80 per cent of women.

The species *L. plantarum* and *L. pentosus* are genotypically closely related and show highly similar phenotypes. The two share >99 per cent identity value in their 16S rDNA sequence[15]. As specificity of species-specific primers of *L. plantarum* was not checked towards *L. pentosus*, or another closely related species *L. paraplantarum*[8]; 16S rDNA sequencing was performed as a confirmatory test.

An observation of interest was the existence of different strains amongst various species of lactobacilli. Strains presenting a common band in species-specific PCR had different RAPD profiles. This was in agreement with the earlier findings[12,14].

In conclusion, our study showed the lactobacilli present in the healthy vagina of Indian women. However, more studies in other parts of the country are indicated to confirm the findings. This information might be beneficial for the development of probiotic tablets seeking to replenish the missing lactobacilli for reproductive health of women.

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