Predominant *Lactobacillus* species Identification from Healthy and Unhealthy Saudi Women by Molecular Techniques

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In contrast to the Lactobacillus-dominated healthy genital organ, bacterial infection of female genital organwas associated with a range of negative outcome acquisitions including genital transmitted diseases, preterm births, and pelvic inflammatory. So long, laboratory diagnosis of bacterial infection of women reproductive organ depended on Gram-stained swabs and microscopic observation. In this study, We described an easy molecular method for the identification of Lactobacilli species by amplified primers : (LactoF, LactoR, LcrisF, LcrisR, LjensF, LjensR, LgassF, LgassR, LinersFLinersR) of Lactobacilli specie specific. Sixty swab samples from healthy women and sixty from infected; ranging their ages from 20- 40 years were donated freshly by Alhabib Hosbital and Malaz Clinic, Riyadh Saudi Arabia during 2012 respectively. Microbial DNA was extracted by two methods, extraction kit and boiling. PCR mixtures were prepared. This study was aimed to identify Lactobacillus species from healthy and unhealthy women.Our results did not provide support for the findings of YAN Dong-hui et al in Chinese women and Elahe Motevaseli et al of Iranian healthy and unhealthy women that L. crispatus, L. gasseriand L. jenseniiwere only dominant spices in healthy women. In contrast to their findings, we found that L. crispatus, L. gasseri, L. iners were dominant in Saudi healthy women although their dominant numbers were significantly different, while, L. jensenii was dominant only in unhealthy women.

Key words: Lactobacilli, Pelvic inflammatory, Female genital organ, Molecular technique.

Lactobacilli are the predominant bacteria in the lower genital tract in women of reproductive age. The presence of these bacteria is a prerequisite for a healthy vaginal condition. The genus Lactobacillus is the largest group among the Lactobacteriaceae, and contains around 140 species and 30 subspecies (Boris S, Barbes *et al*; 2000 & Claesson, M.J.D. *et al*; 2007). These numbers are constantly being revaluated on the basis of modern molecular biology methods and whole genome-based techniques (Makarova, K., *et al*; 2006 & Felis, G.E. *et al*; 2007). Since then, based again on 16S rDNA sequences, it was proposed to divide, the Lactobacillus species into

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five groups, namely L. acidophilus, L. salivarius, L. reuteri, L. buchneri and L. plantarum (Schleifer, K.H. et al; 1995). However, these classifications have generally been considered as unsatisfactory and also the use of 16S rRNA genes as phylogenetic markers has been criticized (Claesson, M.J.D. et al; 2007). New proposals for the classification of the Lactobacilli species claim that the genus could be divided into seven or eight groups (Dellaglio F. et al; 2005 & Hammes, W. P. et al; 2006). As complete genome sequences become available, the high diversity of Lactobacillus has also been suggested to require the creation of new, sub generic divisions. Lactobacilli act by restraining the growth of pathogenic microorganisms via several mechanisms of which the lactate metabolite is considered one of the major factors, keeping the pH below 5. (Witkin S.S.

et al; 2007 & Claesson, M. J., D. et al; 2007). Abnormal vaginal microbiota, such as bacterial vaginosis (BV), the most prevalent vaginal disorder in women of child-bearing age, is associated with an increased risk of gynaecologic and obstetrical complications, such as postoperative infections, spontaneous abortion, and preterm birth (Ness R B et al; 2004 & Leitich H. et al; 2007). BV is also associated with increased risk of acquisition of sexually transmitted infections (Nelson D.B. et al, 2008). BV may also be asymptomatic. In addition, a disturbed non-BV microbiota has also been associated with pregnancy complications. In BV the Lactobacillus-dominated micro biota has been replaced by high numbers of anaerobic bacteria. From a microbiological point of view, BV is an enigma and the factors that initiate the transformation to an abnormal vaginal micro biota are not known. The diagnosis of BV is based on pH, and fresh wetmount microscopy, or microscopy of Gram-stained vaginal smears. The methods do not identify specific microorganisms. In order to understand more about the mechanism behind the change of a Lactobacillus-dominated micro biota to an abnormal one, the bacterial community needs to be characterized, and their relation to host innate immune factors investigated. It is necessary to understand the relationship between bacterial patterns and different clinical conditions or risks. In practice, this information can help to develop effective treatment of unwanted vaginal conditions due to abnormal microbiota and provide prophylactic screening to reduce gynecologic and obstetrical complications. The human vaginal epithelium prevents colonization by exogenous microbes and their entrance into deeper tissue. At the same time, it supports luminal commensal bacteria by providing suitable conditions for their growth. The vaginal surface is kept moist by a fluid that is a transudate through the vaginal epithelium and from the cervical mucus with additional fluids from the endometrium, uterine tubes, and vestibular glands. The vaginal secretion is a mixture of several components, including ions (Na⁺, Ca²⁺, Cl⁻) proteins/peptides, glycoproteins, lactic acid, acetic acid, glycerol, urea, and glycogen, which vary depending on the absolute levels and ratios of estrogens and progesterone, sexual stimulation and the status of microbiocenosis. Additionally, the vaginal secretion contains

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exfoliated cells, which under the estrogen stimulation, is predominated by cells of the superficial layer, while, in the progesterone phase, cells of the intermediate layer become more frequent⁹.

MATERIALS

MRS agar and MRS broth ,Taq PCR Master Mix. **Primers**

(Lactobacillus – L. jensenii - L.crispatus				
-L.gasseri – L.iner primers)				
AGCGAGCGGAACT				
AACAGATTTAC	L.crisF			
AGCTGATCATGCGAT				
CTGCTT	L.crisR			
AAGTCGAGCGAGCT				
TG CCTATAGA	L. jensF			
CTTCTTTCATGCGA				
AAGTAGC	L. jensR			
AGCGAGCTTGCCT				
AGATGAATTTG	L, gassF			
TCTTTTAAACTCTA				
GACATGCGTC	L. gassR			
CTCTGCCTTGAAGA				
TCGGAGTGC	L. inersF			
ACAGTTGATAGGCA				
TCATCTG	L. inersR			
	tobacillus – L. jensenii - L Liner primers) AGCGAGCGGAACT AACAGATTTAC AGCTGATCATGCGAT CTGCTT AAGTCGAGCGAGCT TG CCTATAGA CTTCTTTCATGCGA AAGTAGC AGCGAGCTTGCCT AGATGAATTTG TCTTTTAAACTCTA GACATGCGTC CTCTGCCTTGAAGA TCGGAGTGC ACAGTTGATAGGCA TCATCTG			

5x TBE buffer (89mM Tris, pH 8.3; 89mM boric acid; 2Mm EDTA).

ethidium bromide, Big dye buffer (enzyme labeled DNTP s), Qiagene Dye Ex column

METHODS

Isolation of *Lactobacilli* from female genital organ:

MRS broth and MRS agar were used as isolation media for *Lactobacilli* species from swabs of urogenital female. A sterile swab was rolled over the high vaginal wall and placed in sterile screw cap tubes containing MRS broth were cultured on agar plates then from each plate pure colonies were selected from each plate and cultured individually in MRS broth and stored in 20% glycerol at -70°C

DNA Extraction

Total DNA of the samples were extracted using Charge Switch® gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, Calif, USA), Genomic DNA was isolated from 0.5 ml aliquots of the cell suspensions using a two-step cell lysis procedure. First, suspend the cell pellet in 100 µl of Suspension Buffer (R4) containing RNase A and 5 µl of lysozyme solution (50 mg/ml) by vortexing. Ensure that the cells are evenly distributed. Followed by Incubation of the samples for 10 minutes at 37°C. After that added 500 µl Lysis Buffer/Proteinase K, mixtured and incubate the samples for 10 minutes at 55°C. Added 40 µl Charge Switch® Magnetic Beads to the samples and mixed well, then added 300 µl Binding Buffer (B8) and mixed using a vortex mixer, and incubated at room temperature until the beads have formed a tight pellet. Then, discard the supernatant without disturbing the pellet of beads. After that, added 1 ml Wash Buffer twice to the tube and mixed the sample without forming bubbles. Then, discard the supernatant without disturbing the pellet. And then, added 200 µl Elution Buffer (E5; 10 mMTris-HCl, pH 8.5) to the tube and mixed the samples, Incubate at room temperature for 5 minutes, removed the supernatant containing the DNA to a sterile micro centrifuge tube, Stored the purified DNA at -20°C.

Following the specifications provide by the manufacturer. DNA quality was estimated by electrophoresis in 1% agarose gels in TBE buffer (89 mMTris, pH 8.3; 89 mM boric acid; 2 mM EDTA) and staining with 2 μ g/mL ethidium bromide. **Boiling Method**

Washed swab samples in 200µl distilled water and transferred to sterile microcenterifuge tube and sealed with parafilm to prevent release of contents and contamination of samples then boiled at 100°C for 15 min.

After centrifugation at 1000 rpm for 5 min, about $100\mu l$ of supernatant was removed and was used for PCR.

PCRAmplification

PCR mixtures were prepared with 4 μ l of 5X FIREPOL Master Mix, 2.4 μ l for primers, 5.2 μ l of genomic DNA and sterile filtered mille water to final volume of 20 μ l. PCR amplification sub was as follows: denaturation at 95°C for 3min, 30 cycles was applies as follow: 95°C for 30s, 55- 60°C (depending on each procedure) for 1min and 72°C for 1 min in final extension step at 72°C for 5 min. **Gel electrophoresis**

Following PCR, 8µl of the reaction mixture was mixed. The mixture was electrophoresed in 2% agarose gel in Tris-borate-EDTA buffer (TBE) at 60 V for 100 min or was continued until the loading buffer tracking dye approached the front of the running gel. The amplified DNA bands were visualized following ethidium bromide staining and photographed under UV light. Ladder: 100bp (Invitrogen[™], Life Technologies) DNA size Marker was used to mark molecular masses of PCR amplicons.

RESULTS

Lactobacilli were isolated from swab samples by using DE MAN, ROGOSA, and SHARPE (MRS) broth and agar media. From each plate individual pure cultured colonies were selected and overnight shake at 37 in MRS broth and stored in 20% glycerol at -70°C.

Name	Sequence (5'-3')	Target	Annealing Temp. °C
LactoF	TGGAAACAGRTGCTAATACCG		
LactoR	GTCCATTGTGGAAGATTCC	Lactobacillus	60
LctrisF	AGCGAGCGGAACTAACAGATTTAC		
LcrisR	AGCTGATCATGCGATCTGTT	L. crispatus	60
LjensF	AAGTCGAGCGAGCTTGCCTATAGA		
LjensR	CTTCTTTCATGCGAAAGTAGC	L. jersenii	60
L gassF	AGCGAGCTTGCCTAGATGAATTG		
LgassR	TCTTTTAAACTCTAGACATGCGTC	L. gasseri	55
LinersF	CTCTGCCTTGAAGATCGGAGTGC		
LinersF	ACAGTTGATAGGCATCATCTG	L. iners	55

Table 1. PCR primers

Specie specific Lactobacillous primers used in this study

Pure total genomic DNA of each sample was extracted from 0.5 ml aliquots of each sample using Charge Switch® gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, Calif, USA), Genomic. Removed the supernatant containing the DNAto a sterile micro centrifuge tube, stored the purified DNA at -20°C. Electrophoresis in 1% agarose gels

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Healthy women

Fig. 1. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)

Latobillus crispatus primer: F : ACG CAG CGG AAC TAA CAG ATT TAC R : AGC TGA TCA TGC GAT CTG CTT



Healthy women

Fig. 3. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)
Latobillus iners primer:
F : CTC TGC CTT GAA GAT CGG AGT GC

R : ACA GTT GAT AGG CAT CAT CTG

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in TBE buffer showed under UV light good quality of DNA. Boiling Method of DNA isolation directly from swab samples by washing the samples in 200µl distilled water and transferring to sterile microcenterifuge tube then boiling at 100°c for 15 min and sealed with Para film to prevent release of contents, contamination and evaporation of



Infected women

Fig. 2. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)

Latobillus crispatus primer: F : ACG CAG CGG AAC TAA CAG ATT TAC R : AGC TGA TCA TGC GAT CTG CTT



Infected women

Fig. 4. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)

Latobillus iners primer:

- F : CTC TGC CTT GAA GAT CGG AGT GC
- R : ACA GTT GAT AGG CAT CAT CTG

samples. After centrifugation at 1000 rpm for 5 min, about 100μ l of supernatant was removed then 10μ l of the 100 μ l containing the released DNA was electrophoresis using 1% of agarose gel in 1X TBE buffer and was visualized under UV light showed good quality of DNA.

Primers used in the amplification of *Lactobacilli* species were shown in (Table 1.) PCR



Healthy women

Fig. 5. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)

Latobillus gasseri primer: F : AGC GAG CTT GCC TAG ATG AAT TTG R : TCT TTT AAACTC TAG ACA TGG GTC



Healthy women

Fig. 7. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)

Latobillus jensenii primer: F : AAG TCG AGC GAG CTT GCC TAT AGA R : CTT CTT TCA TGC GAA AGT AGC mixtures were prepared with 4 μ l of 5X FIREPOL Master Mix, 2.4 μ l for primers, 5.2 μ l of genomic DNA and sterile filtered mille water to final volume of 20 μ l. PCR amplification program was as follows: denaturation at 95°C for 3min, 30 cycles were applied as follow: 95°C for 30s, 55- 60°C (depending on each procedure) for 1min and 72°C for 1 min in final extension step at 72°C for 5 min. The amplicons for



Infected women

Fig. 6. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen)
Latobillus gasseri primer:
F : AGC GAG CTT GCC TAG ATG AAT TTG

R : TCT TTT AAACTC TAG ACA TGC GTC



Infected women

Fig. 8. Electrophoresis on a 2% agarose gel of PCR product. Lane M, Molecular weight marker (100 bp, Invitrogen) Latobillus jensenii primer:

F : AAG TCG AGC GAC CTT GCC TAT AGA

R : CTT CTT TCA TGC GAA AGT AGC

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each primer specific Lactobacilli species in healthy and unhealthy women were shown in figures (1-8). Figure one shows five PCR product < 200 bp and > 100bp of L. crispatus in healthy women while figure two shows six PCR product<200bp and >100bp of L. crispatus in unhealthy women. Figure three shows eight PCR product < 200bp and > 100bp of L. iners in healthy women while figure four shows thirteen PCR product<200bp and >100bp of L. iners in unhealthy women. Figure five shows zero PCR product of L.jensenii in healthy women while in figure six shows one PCR product <200bp and > 100bp of *L. jensenii* in unhealthy women. Figure seven shows one PCR products of L. gasseri in healthy women while in figure eight shows also one PCR product of L. gasseri<200bp and > 100 bp in unhealthy women.

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DISCUSSION

The health status of the human female urogenital tract is largely a function of its micro flora. Lactobacilli have been demonstrated to dominate the normal urogenital environment. Lactobacilli play a key role in providing protection against invasion of pathogens or against overgrowth of potentially pathogenic species among the normal flora through the production of hydrogen peroxide, bacteriocins, and lactic acid (Vitali B. et al; 2007). In the past, Lactobacilli flora of healthy women was believed to be dominated by L. acidophilus and L. fermentum, followed by L. brevis, L. jensenii, L. casei, and other species (K. C. Anukamet al; 2005). But recent studies using molecular methods indicated that L. crispatus, L. iners, and L. jensenii were the most common Lactobacilli isolates of healthy vaginal flora (K. C. Anukamet al; 2007). In this study, PCR method was used to identify Lactobacillus species flora in health and infected Saudi women. We found that L. iners, L. crispatus, L. gasseriand L. jensenii were the most common species in health Saudi women. Our results are in accordance with studies conducted by Akunam et al. (Anukam KC. et al;2006) predominant Lactobacillus species: L.crispatus, L. gasseri, L. jensenii, and L. iners were identified in healthy and unhealthy women though their respective percentage was different. Though genital tract dominated by Lactobacilli appears to protect the host against some infections,

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it does not fully prevent colonization by other species. Pathogens are still able to coexist with these commensal organisms, as described by Burton and Reid (Burton JP et al; 2002). We also observed an apparent increase in persistence for L.crispatus and L.iners isolates over L. jensenii and L.gasseriisolated from healthy and infected women. L. jensenii was only isolated from infected women whereas the other predominant Lactobacillus species (L.crispatus, L.gasseri and L.iners) were isolated from healthy and infected women both. Most prevalent Lactobacillus species was *L.iners* which can be detected *L.iners* at high levels in healthy and infected women. It has been postulated that this may be because L. iners may be better adapted to the conditions associated with infection (Cosgrove N. et al; 2009). Alternatively, the observations could result from relative resistance of L. iners to unknown factors that lead to the demise of other Lactobacillus species during the onset of infection, or to a relative lack of antagonism of L. iners to the infection associated anaerobes, so that their dominance predisposes the individual to acquiring infection. Typically, one or two Lactobacilli species are predominant. For example, L.crispatus and L.jensenii were the most common genera for white women, while L. crispatus and L. gasseri were more common in Japanese women. Recently, a study from China reported that L.crispatus, L.iners and L.gasseri were the most common genera in Chinese women. In our study, L. iners was widely present in high loads in both healthy and unhealthy women. Lactobacillus species have been reported to differ in their ability to exclude other bacteria, and L.iners is the species that most often co-exists with other 'less-healthy' microorganisms. The presence of L.iners, one of the common Lactobacillus species in the genital tract of women may be correlated with tract colonization by this infection related bacteria.

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REFERENCES

- 1. Boris S, Barbes C., Role played by *Lactobacilli* in controlling the population of vaginal pathogens. *Microbes Infect* 2000; **2**(5):543-546.
- Claesson, M. J., D. van Sinderen, and P. W. O'Toole., The genus *Lactobacillus*: a genomic basis for understanding its diversity. *FEMS Microbial. Lett.* 2007; 269:22–28.
- Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev. Comparative genomics of the lactic acid bacteria. ProcNatlAcadSci U S A 2006; 103:15611–15616.
- 4. Felis, G.E., Molenaar, D., Dellaglio, F., and van HylckamaVlieg, J.E.T., Dichotomy in postgenomic microbiology.*Nature Biotechnol*.in press 2007.
- Schleifer, K.-H., and Ludwig, W., Phylogeny of the genus *Lactobacillus* and related genera. *System Appl. Microbiol* 1995; 18: 461–467.
- Dellaglio F., and Felis G.E., Taxonomy of Lactobacilli and bifidobacteria. In: Probiotics and Prebiotics:Scientific Aspects, pp. 25–50. Edited by G.W. Tannock, Caister Academic Press, Norfolk, UK, 2005.
- Hammes, W. P. &Hertel, C., The genera Lactobacillus and Carnobacterium. In The Prokaryotes, 3rd edn, vol. 4, pp. 320– 403.Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer&E. Stackebrandt. New York: Springer, 2006.
- Witkin S.S., Linhares L.M., Giraldo P., Bacterial flora of the female genital tract: function and immune regulation. *Best Practice & Research Clinical Obstetrics and Gynaecology*. 2007; 21(3): 47-354.
- 9. Ness RB, Hillier SL, Kip KE, Soper DE, Stamm CA, McGregor JA, Bass DC, Sweet RL,Rice P, Richter HE., Bacterial vaginosis and risk of pelvic inflammatory disease.*Obstet Gynecol*, 2004; **104**(4): 761-769.

- Leitich H, Kiss H., Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Pract Res Clin Obstet Gynaecol*, 2007; 21(3):375-390.
- 11. Nelson DB, Bellamy S, Nachamkin I, Ruffin A, Allen-Taylor L, Friedenberg FK., Characteristics and pregnancy outcomes of pregnant women asymptomatic for bacterial vaginosis.*Matern Child Health J*, 2008; **12**(2): 216-222.
- Vitali B, Pugliese C, Biagi E, Candela M, Turroni S, Bellen G, *et al.*, Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis, or no infection, analyzed by PCR-denaturing gradient gel electrophoresis and real-time PCR. *Appl Environ Microbio* 2007; **I73**(18): 5731-5741.
- K. C. Anukam, E. O. Osazuwa, I. Ahonkhai, and G. Reid., "16S rRNA gene sequence and phylogenetic tree of *Lactobacillus* species from the vagina of healthy Nigerian women," *African J. Biotechnol*, 20 05; 4(11): 1222–1227.
- 14. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P, *et al.*, Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women.*ISME J* 2007; 1(2):121-133.
- Anukam KC, Osazuwa EO, Ahonkhai I, Reid G., *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin city, *Nigeria. Sex Transm Dis.* 2006; 33(1): 59–62.
- Burton JP, Reid G, Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. *J Infect Dis.* 2002; 186(12): 1770-1780.
- Cosgrove N, Holzman C, Marsh TL. (2009). Temporal Shifts in Microbial Communities in Nonpregnant African-American Wertz J, Isaacs Women with and without Bacterial Vaginosis. *InterdiscipPerspect Infect Dis* 2008: 181253.