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Sequence of *Lactobacillus inners* PCR Product: Dominant Species in Healthy and Unhealthy Saudi Women

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Bacterial infection of female urogenital is associated with a range of negative outcomes, in contrast to the *Lactobacillus*-dominated healthy genital organ. So long, *Lactobacillus* diagnosis of sample from women vaginal depended on Gram-stained swabs and microscopic observation.This study describes in easy molecular method for the identification of *Lactobacilli inners* by amplifying primers: [LinersF and LinersR] of *Lactobacilli* specie-specific sequence. Sixty swab samples from healthy women and sixty from infected ranging their ages from 20 – 49 years were donated freshly by AlhabibHospital and Malaz clinic, Riyadh, Saudi Arabia during 2012 respectively. DNA was extracted by using extraction kit andPCR mixtures were prepared. The PCR productsof *Lactobacilli inners*were directly sequenced on an Applied Biosystem 3130 x 1 Genetic Analyzer (Applied Biosystems , Hitachi High-Technologies corporation Tokyo-Japan).This study was aimed to identify the predominance of*Lactobacillus inners*species from healthy and unhealthy Saudi women.This result provided support for the findings of (Najla Qalit Ammash, 2013)that *L.inners* were dominant species in Saudi healthy and unhealthy women in contrast toYAN Dong-hui *et al* of Chinese women and Elahe Motevaseli *et al* of Iranian healthy and unhealthy women. In this study was found that the *sequence* of PCR product of *L. inners* of healthy and unhealthy Saudi women matched, indicating that the*inners* were the most dominantspecies in the Saudi population.Their dominant numbers might significantly be different from country to country therefor; we propose the need of further research on the subject.

Key words: Lactobacilli, healthy, unhealthy women,female,genital, organ.

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 (Zegels,G *et al*; 2010). New proposals for the classification of the lactobacilli species claim that the genus could be divided into seven or eight groups. 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. Abnormal vaginal micro biota, such as bacterial vaginitis (BV), the most prevalent vaginal disorder in women of child-bearing age, is associated with an increased risk of gynecologic and obstetrical complications, such as postoperative infections, spontaneous abortion, and preterm birth (Mashburn J. 2006). BV is also associated with increased risk of acquisition of sexually transmitted infections. BV may also be asymptomatic. IN addition, a disturbed non-BV micro biota 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

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abnormal vaginal micro biota are not know (Ness RB. *et al*; 2004). The diagnosis of BV is based on pH, and fresh wet mount 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 (Gilbert G.G. *et al*; 2007). 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 micro biota and provide prophylactic screening to reduce gynecologic and obstetrical complications. Infections of the lower genital tract are classified according to the site of symptoms and clinical findings, e.g., vaginitis, and cervicitis. Lower genital tract complaints, such as abnormal discharge, vaginal itching and vaginal burning, among women may be the results of bacterial vaginitis (BV), vulvovaginal candidiasis, Trichomoniasis, gonorrhea and Chlamydia infections (Gilbert G.G. *et al*; 2007). BV is the most common cause of abnormal discharge in women of reproductive age. It is a polymicrobial vaginal disorder with a heavily disturbed vaginal microbiota, where the Lactobacillus - predominant microbiota is replaced by an overgrowth of anaerobic bacteria. This condition is most often not associated with clinical signs of inflammation (such as vaginal wall erythema, and leukocytosis), thus the term "vaginosis" is used instead of "vaginitis Bacterial vaginitis, also called aerobic vaginitis, is not a common condition and sometimes is confused with BV (Stokholm J. *et al*; 2012). Group B streptococci, alphahaemolytic streptococci, *Escherichia coli* and *Staphylococcus aureus* may cause bacterial vaginitis. The normal vaginal micro biota is a unique and dynamic system and continually fluctuates under the environmental changes and physiological conditions. The vagina of healthy fertile women harbours an extensive number of bacteria, of which lactobacilli predominate. Lactobacilli differ greatly in morphology between various species; Lactobacilli are the most well-known markers of normal vaginal flora. Their ability to produce an acid pH in the vagina (mainly due to the acidification enzyme hydrogen peroxidase) and bacteriocins that kill off other bacteria makes them prime candidates for the surveillance of vaginal health (Wilks M.*et al*; 2004). Lactobacilli metabolise glycogen released from epithelial cells. Glycogen is degraded into glucose, which is fermented to lactic acid via pyruvate. Lactic acid and the low pH of vaginal secretions have been shown to exert antimicrobial activity against non-resident bacteria (Sabina Cauci. *et al*; 2002). The genus Lactobacillus is the largest group among the Lactobacteriaceae, and contains around 140 species and 30 subspecies. These numbers are constantly being revaluated on the basis of modern molecular biology methods and whole genome-based techniques. Since then, based again on 16S rDNA sequences, it was proposed to divide, the Lactobacillus species into five groups, namely *L. acidophilus, L. salivarius, L. reuteri, L. buchneri* and *L. plantarum*. However, these classifications have generally been considered as unsatisfactory and also the use of 16S rRNA genes as phylogenetic markers has been criticized (Collins, M.D. *et al*; 1991).

The vaginal epithelial structures, as well as the micro biota, change considerably from childhood to menopause. At birth, the neonatal vaginal epithelium is rich in glycogen, due to maternal estrogen. Thus, the infant vagina is colonised by lactobacilli within the first 24 hours after birth, acquired from the maternal birth canal (Carlsson, J. *et al*; 1975) Several weeks later, when the level of estrogen has decreased, the vaginal epithelium becomes thin and atrophic with a low glycogen levels. Gram-positive cocci and bacteria other than lactobacilli become predominant, a condition, which continues until the puberty. During menstruation, non-Lactobacillus species appear to increase in number, while the lactobacilli decrease or stay approximately the same number. During pregnancy, a Lactobacillus-dominant micro biota is strengthened by the increased estrogen levels, however, at the same time, the incidence of vulvovaginal candidiasis increases, compared with non-pregnant women. The reason for the increase has been proposed to be somewhat suppressed cell mediated immunity in pregnant women leading to an increased susceptibility to pathogens such as: *C. albicans*. At menopause, the prevalence of lactobacilli is decreased, due to the low estrogen

levels, and the vaginal pH is increased. The vaginal micro biota of postmenopausal women is similar to that in the pubertal period (Patta MC. *et al*; 2008). Lactobacilli, yeasts and BV-associated bacteria are a less common component of the vaginal micro biota in postmenopausal women than in women of reproductive age, while *E. coli* is recovered at higher frequency. Thus, estrogens have a decisive effect on the composition of the lower genital tract micro biota. Antimicrobial agents can adversely affect the vaginal lactobacilli. Lactobacilli have variable susceptibility to cephalosporin, but sensitive to penicillin. In contrast, vancomycin, doxycycline and metronidazole are inactive against lactobacilli. Clindamycin vaginal cream, used for treatment of BV is also active against lactobacilli. There are contradictory reports on the effects of contraceptives on the vaginal micro biota. Some studies have reported no major changes in the levels of aerobic and anaerobic bacteria in oral pill users, intrauterine device (IUD) – users and no users. Accurate genomic methods are needed in order to define the composition of the Lactobacillus microbiota in the vagina, not only for treatment of infectious diseases but also to establish the normal Lactobacillus micro biota in different settings (Donati L. *et al*; 2010). In the last decade molecular techniques, including polymerase chain reaction-based and other genotyping methods have become increasingly important for species identification and for differentiation of Lactobacillus isolates.

MATERIALS AND METHODS

MRS agar and MRS broth

Lactobacillus MRS Agar is recommended for cultivation of *Lactobacillus specie*s.

Composition

Ingredients	Gms / Litre
Proteose peptone	10.000
Beef extract	10.000
Yeast extract	5.000
Dextrose	20.000
Polysorbate 80	1.000
Ammonium citrate	2.000
Sodium acetate	5.000
Magnesium sulphate	0.100
Manganese sulphate	0.050
Dipotassium phosphate	2.000
Agar	12.000
Final pH (at 25°C)	6.5±0.2

Isolation of vaginal lactobacilli

MRS broth and MRS agar were used as isolation media for vaginal lactobacilli. Although both of them are suitable media for lactobacilli but other bacteria can grow.None lactobacilli grew on MRS agar showed different in morphology and physiology such as cocci shape, catalase negative or spore –forming. *Lactobacilli* are known as facultative or anaerobe, most of selected isolates of lactobacilli can survive when exposed to oxygen in ambient air. It is indicated that these isolates of tolerate to oxygen and it is easier to culture these bacteria than obligate anaerobe ones. A sterile swab was rolled over the high vaginal wall and placed in sterile screw cap tubes containing MRS broth were selected from each plate and cultured individually in MRS broth and stored in 20% glycerol at –70°C.

Table 1. Revese and forward primer of *L. inners*

Name	Sequence(5'-3')	Target	Annealing Temp. (°C)
LinnersF	CTCTGCCTTGAAGATCGGAGTGC	Lactobacillus species	55
LinnersR	ACAGTTGATAGGCATCATCTG		

Table 2. Cycle Sequencing Protocol

Temperature	Time	Cycle
98ºC	Ramp 1 ºC/Sec	
98ºC	5 min	
98ºC	Ramp 1 ºC/Sec	35 cycle
98ºC	10 Sec	
60 ºC	Ramp 1 ºC/Sec	
60 ºC	4 min	
4 ºC	Ramp 1 ºC/Sec	
4 ºC	Forever	

DNA Extraction and PCR Amplification Conditions

DNA Extraction

Total DNA of the vaginal samples was extracted using ChargeSwitch® 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, Resuspend the cell pellet in 100 µl of Resuspension 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 Incubate the sample for 10 minutes at 37°C. after that add 500 µl Lysis Buffer/ Proteinase K mixture and Incubate the sample for 10 minutes at 55°C. After that add 40 µl ChargeSwitch® Magnetic Beads to the sample and mix well, then add 300 µl Binding Buffer (B8) and mix using a vortex mixer, and incubate at room temperature until the beads have formed a tight pellet. then, discard the supernatant without disturbing the pellet of beads add 1 ml Wash Buffer twice to the tube and mix the sample without forming bubbles. Then, discard the supernatant without disturbing the pellet. And then, add 200 µl Elution Buffer (E5; 10 mM Tris-HCl, pH 8.5) to the tube and mix the sample, Incubate at room temperature for 5 minutes. Removed the supernatant containing the DNAto a sterile micro centrifuge tube. Store 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 mM Tris, pH 8.3; 89 mM boric acid; 2 mM EDTA) and staining with 2 μg/mL ethidium bromide.

PCR Amplification Conditions:

PCR was carried out using one primers. PCR mixtures were prepared with 4 µl of 5X FIREPOL Master Mix , 2.4 µl for primers , 5.2 µl of genomic DNA and sterile filtered mille water to final volume of 20 µl . PCR amplification sub was as follows: denaturation at 95ºC for 3min, 30 cycles was applies as follow: 95ºC for 30s, 55- 60ºC (depending on each procedure) for 1min and 72ºC for 1 min in final extension step at 72ºC 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. Hundred bp Ladder (Invitrogen™, Life Technologies) DNA size Marker was used to mark molecular masses of the PCR product.

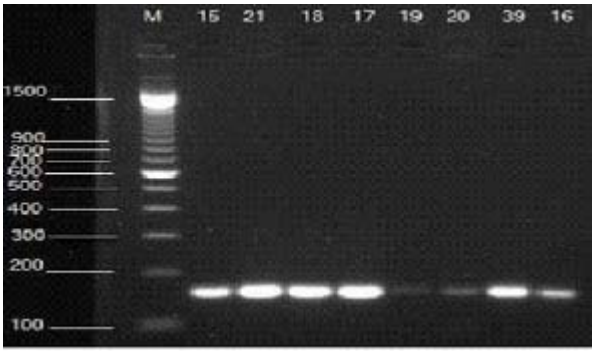
DNA sequencing

PCR products were purified using a Qiagene dye EX column Gel Extraction Kit (QIAGEN®, DyeEx™) according to the manufacturer's instructions.The products were directly sequenced on an Applied Biosystem 3130 x 1 Genetic Analyzer (Applied Biosystems,Hitachi High-Technologies corporation Tokyo-Japan) using forward or reverse primer used in PCR reaction according to the manufacturer's instructions.

Ambiguous and incorrect called bases were manually corrected using Chromas Lite software, version 2.01 (Technelysium Pty Ltd.).

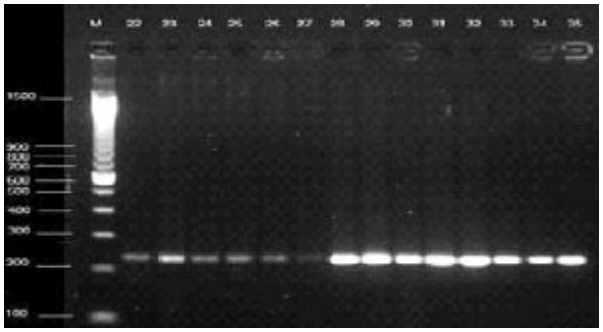
RESULTS AND DISCUSSION

Polimerase chain reactinCR was carried out using one primers see. The amplified DNA bands were visualized following ethidium bromide staining and photographed under UV light. Hundred bp Ladder (Invitrogen™, Life Technologies) DNA size Marker was used to mark molecular PCR was carried out using one primer. The amplified DNA bands were visualized following ethidium bromide staining and photographed under UV light. Hundred bp Ladder (Invitrogen™, Life Technologies) DNA size Marker was used to mark molecular masses of the PCR product. PCR products were purified using a Qiagene dye EX column Gel Extraction Kit (QIAGEN®, DyeEx™) according to the manufacturer's instructions. The products were directly sequenced on an Applied Biosystem 3130 x 1 Genetic Analyzer (Applied Biosystems,Hitachi High-Technologies corporation Tokyo-Japan) using forward or reverse primer (Table 1) with Cycle Sequencing Protocol (Table 2) and the PCR product (Fig. 1 & 2). By using molecular techniques our results showed that women were dominated by the *Lactobacillus* species that the healthy and unhealthy women are usually colonized by only one or two dominant *Lactobacillus species*, mainly *L. inners*.We found that only one species of *Lactobacillus*was numerically abundant in Saudi women.*L. niners*, PCR product in the healthy and unhealthy women were found 75% out of 120



Healthy Women

Fig. 1. Electrophoresis on a 2% agarose gel of PCR Products
Lane M, Molecular weight marker (100 bp, invitrogen) Lactobacillus iners primer
F: CTC TGC CTT GAA GAT CGG AGT GC
R: ACA GTT GAT AGG CAT CAT CTG



Infected women

Fig. 2. Electrophoresis on a 2% agarose gel of PCR Products
Lane M, Molecular weight marker (100 bp, invitrogen) Lactobacillus iners primer
F: CTC TGC CTT GAA GAT CGG AGT GC
R: ACA GTT GAT AGG CAT CAT CTG

individuals. In this study *L. inners* was the most common specie of *Lactobacillus* in women; the exclusion of other species is in keeping with the theory of "competitive exclusion", and the superior ability of *L. inners* to compete with other bacteria for vaginal resources, a survival strategy known as "bacterial interference". This result provided support for the findings of (Najla Qalit Ammash) that L.inners were dominant species in Saudi healthy and unhealthy women in contrast to YAN Dong-hui *et al* of Chinese women and Elahe Motevaseli *et al* of Iranian healthy and unhealthy women. In this study was found that the sequence of PCR product (Fig. 3 & 4) of *L. inners* of healthy and unhealthy Saudi women matched, indicating that the inners were the most dominant species in the Saudi population. Their dominant numbers might significantly be different from country to country therefor; we propose the need for further research on the subject.

DISCUSSION

Lactobacilli are ubiquitous in the environment. They colonize plants, animals and humans (Collins *et al*; 1991). In the human body, lactobacilli may colonize three anatomic regions: the oral cavity, the intestines and the vaginal tract. Although the lactobacilli that inhabit the vaginae of mothers contaminate the infants' mouth during delivery, they do not appear to colonize the oral cavity (Carlsson and Gothefors 1975) or the intestines of the infants after birth. It is unknown, however, whether food or the environment could be a source of lactobacilli that colonize humans.The composition of vaginal flora is the focus of interest

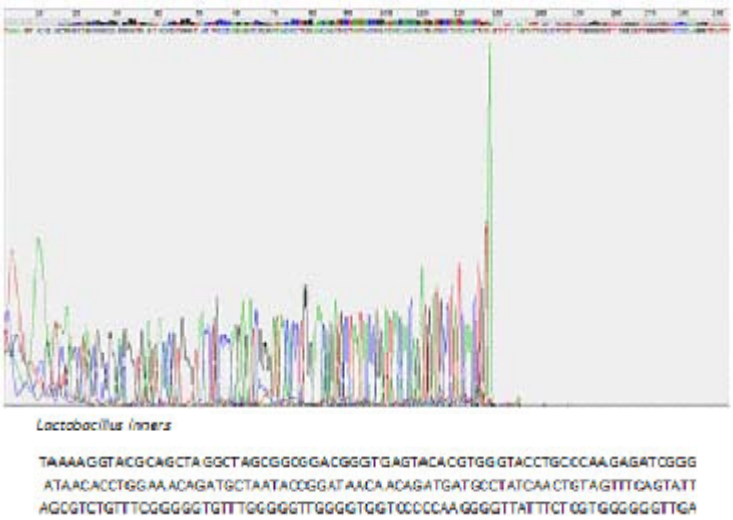


Fig. 3. DNA sequence Analysis from PCR product for *Lactobacillus inners* in infected women
F: CTC TGC CTT GAA GAT CGG AGT GC
R: ACA GTT GAT AGG CAT CAT CTG

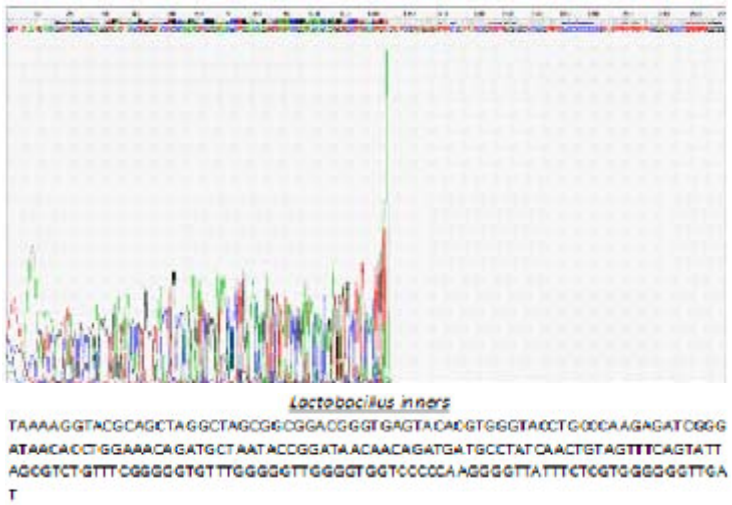


Fig. 4. DNA sequence Analysis from PCR product for *Lactobacillus inners* in healthy women
F: CTC TGC CTT GAA GAT CGG AGT GC
R: ACA GTT GAT AGG CAT CAT CTG

of recent investigations because of its importance to women's reproductive organ and general health (Patta MC.*et al.,* 2008). Vaginal lactobacilli metabolize glycogen secreted by the vaginal epithelia, in turn producing lactic acid, which is largely responsible for the normal vaginal pH being acidic (< 4.5) (Donati L.*et al.* 2010). The predominant species of lactobacilli maintain a low pH through their fermenting activity which protects the area against the invasion of undesirable microorganisms (Pascual LM. *et al.,* 2006).The acidic environment of a healthy vagina is not permissive for growth of many potential pathogens (Donati L.*et al.* 2010) . The healthy microbiota of the lower genital tract in women predominantly consists of *Lactobacillus* spp., with *Lactobacillus crispatus*, *Lactobacillus jensenii* and *Lactobacillus iners* being the most prevalent species. It is generally accepted that these bacteria form a critical line of defense against potential

pathogens. The symbiotic relationship between vaginal lactobacilli and their human host is modulated by the hormones circulating in a woman's body, whichstimulate the vaginal epithelia to produce glycogen (Hay p. *et al;* 2005). Vaginal mucosal microfloras are typically dominated by Gram-positive *Lactobacillus species*, which serve as an important natural barrier against infection (Va´squez *et al.*, 2002; Iqbal & Kaul, 2008).*Lactobacillus* spp. ferment glycogen secreted by vaginal epithelial cells into lactic acid, and colonization by these microorganisms correlates to the low pH in thevagina (Boskey *et al*., 2001 and Rönnqvist *et al*., 2006). Najla Qalit *et al*; 2013 in her conclusion, illustrated that the vagina is a dynamic microbial ecosystem supporting a changing and diverse bacterial population. However, comparative genomic studies of the*Lactobacilli* isolated from the vagina of women with normal micro biota and BV might give a clue of the factors involved in colonization resistance, which may lead to the design of better probiotic products as bacterial replacement therapy. This result of this study provided support for the findings of (Najla Qalit Ammash, 2013) that L.inners were dominant species in Saudi healthy and unhealthy women in contrast to YAN Dong-hui *et al* of Chinese women and Elahe Motevaseli *et al* of Iranian healthy and unhealthy women. In this study was found that the sequence of PCR product of L. inners of healthy and unhealthy Saudi women matched, indicating that the inners were the most dominant species in the Saudi population. Their dominant numbers might significantly be different from country to country therefor; we propose the need of further research on the subject.

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