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Lactobacillus Species Detected by *16S* rRNA Gene Sequence Isolated from the vaginae of Pregnant Women and its relation to preterm labor

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ABSTRACT

Preterm labor is a common and serious obstetric complication that is believed to be multifactorial. Lactobacilli vaginal colonization is proposed as a protective agent against preterm delivery and that women with bacterial vaginosis, with altered vaginal flora in favor of lactobacilli species absence, is a risk factor for increased incidence of preterm labor; however enough controversy is present in available published literature. This study was aimed to evaluate the association between vaginal bacterial composition and the rate of preterm labor in pregnant women. Vaginal swabs form a total of 250 pregnant women were obtained and assessed using the V2-V3 region of the 16S rRNA sequences for an estimation of lactobacilli colonization. These women were followed up for the outcome of pregnancy. Women with predominantly lactobacilli species were significantly less liable for preterm labor than those women who lack lactobacilli species in their vaginal samples, 10.4% versus 64.2% (P<0.001). To evaluate the risk of preterm labor associated with non-lactobacilli bacterial colonization, Odds ratio was estimated and it was 15.39 (95% confidence interval of 7.91- 29.95). This study concluded that the risk of preterm labor is significantly increased with bacterial vaginosis in favor of alteration of vaginal flora with deficient lactobacilli species.

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INTRODUCTION

The cause of preterm birth (PTB) is attributable to multiple factors; however, 50% of cases are of unknown etiology. It has been estimated that 20–40% of cases are either due to a specific disease or an indication based deliveries such as fetal growth restriction and pre-eclampsia (Goldenberg *et al.,* 2008). Intrauterine infection and/or inflammation are believed to be responsible for the rest 25–30% of PTB (Goldenberg *et al.,* 2008). The route by which microorganisms can reach the uterus could be via the bloodstream, retrograde through the fallopian tube or ascending through the vagina and cervix (Goldenberg *et al.,* 2000). The vaginal microbial milieu is not constant throughout a female life. Anaerobic bacteria are dominant before puberty (Farage and Maibach, 2006). The increasing estrogen concentration at puberty causes a rise in glycogen production, the metabolic intermediates of which facilitates lactobacilli colonization of the vagina (Spear *et al.,* 2014). This is explaining vaginal colonization by lactobacilli throughout reproductive years of a woman (R Romero *et al.,* 2014). The reduced level of estrogen at menopause leads to diminished lactobacilli colonization (Hummelen *et al.,* 2011). Lactobacilli are Gram-positive facultative anaerobic bacteria| and their attachment the vaginal lining plays an important role in defense against invading pathogens (Othman *et al.,* 2007). What is the normal vaginal microbiota? There is no

evidence to support an answer to this question; however, most studies suggest lactobacilli dominance (Chaban *et al.,* 2014). Recently, the use of sequencing PCR-amplified universal 16S ribosomal DNA (rDNA) brought about better idea regarding the vaginal microbiota and in summary: Few *Lactobacillus* species are the dominant microorganism in the vagina (Lamont *et al.,* 2011). Probably the differences in sequencing methods and the choice of suitable PCR primers more important features of healthy versus diseased female may be revealed in future studies (Walther-António *et al.,* 2014). Despite the fact that somewhat a little 16S DNA researchers were utilized with samples obtained from pregnant ladies, evidence has been found that the microbiota does change throughout pregnancy. Some authors have proposed that they exist up to 5 different community state types (CSTs) of bacteria (Ravel *et al.,* 2011). L. *iners, L. crispatus*, and *L. jensenii* and/or *L. gasseri* are the dominant species in three of the CSTs (I, II, III), whereas CST IV-A and CST IV-B were proved to possess few abundances of and are mainly formed of *Peptoniphilus*, *Anaerococcus*, *Corynebacterium*, *Finegoldia*, and *Prevotella* (CST IV-A), and *Atopobium, Sneathia*, *Gardnerella*, *Ruminococcaceae*, *Parvimonas*, and *Mobiluncus* (CST IV-B) (Spear *et al.,* 2014). It has been suggested that with increasing gestational age, the dominance of *Lactobacillus* spp. rises, whereas those of anaerobe becoming less (Roberto Romero *et al.,* 2014) .

This study was carried out to evaluate the association between vaginal bacterial composition and the rate of preterm labor in pregnant women.

PATIENTS AND METHODS

DNA is extracted from the bacteria that present in 250 vaginal swabs of an apparently healthy pregnant ladies 20-35 years old chosen after excluding women who have the exclusion criteria in the consultation units of Al-Diwaniya Maternity and Teaching hospital from October 2015 to February 2016, their gestational age is between 10-14 weeks of gestation. The vaginal swabs collected after obtaining verbal consents from the patients, by using Instagene Matrix (Bio-Rad Laboratories, Ontario, Canada), according to manufacturer's instructions.

Exclusion criteria included the following: Primiparous patients, Previous obstetric history of preterm labor, Underweight and obese women, Multiple gestation, Vaginal bleeding, In vitro vertilization (IVF), Women carrying abnormal babies, Females having cervical incompetence and uterine anomalies, Short pregnancy interval (less than 6 months duration), Women with certain medical conditions such as diabetes mellitus, hypertension, and other medical diseases, Women with poor social class, poor antenatal care, domestic violence and smokers.

Preparation of swab

Swabs were strongly ruffled in one mL of phosphate buffered saline (PBS) (pH 7.1) to separate cells. Centrifugation at 10,000 grams for five minutes then re-suspension in PBS followed by another run of centrifugation at 13,000 grams for 3 minutes. The pellets then re-suspended in 200 µl Instagene Matrixwere incubated for 20-30 min in a water bath at 55°C. The samples then were shacked, boiled and re-centrifuged. Then the DNA containing supernatants were kept stored at -20°C.

Polymerase chain reaction (PCR)

The DNA sample's amplification reactions were carried out in 0.2 mL PCR single tube (Diamed, Lab. Supplies, Mississauga, Ontario, Canada) with hinged flat cap in a Thermocycler (Eppendorf Mastercycler). The primers were Lac-1(AGCAGTAGGGAATCTTCCA); Lac-2 (GCCGCCCGGGGCGCGCCCC G GGCGGCCCGGGGGCACCGGGGGATTYCACCGCTACAC) (Invitrogen™, Life Technologies)

The PCR amplification was followed by PCR product was analysis by electrophoresis (Bio-Rad) in 1.5% Ultrapure™ Agarose (Invitrogen, Life Technologies) gel, which were seen by Ultraviolet transillumination and recorded with Polaroid 667 instant film.

Sequencing and phylogenetic analysis

Sequences of the fragments are determined by the automatic Big Dye (dideoxy chain terminator) sequencer ABIPRISM 3730xl, (Sequencing Facility, John P.Robarts Research Institute, London, Ontario). Newly determined sequences will be compared to those available in the V2-V3 region of the 16S rRNA sequences using the GeneBank DNA databases and the standard nucleotide-nucleotide BLAST algorithm. Identities of the relatives are determined on the bases of the highest GeneBank accession number.

Statistical analysis

Data are analyzed by using statistical package for social sciences (SPSS version 23.0). Categorical variables are expressed as number and percentages. Chi-square test was used to study the relationship between vaginal bacterial composition and rate of premature labor in pregnant women. Odd ratio and 95% confidence interval were used to estimate risk. P-value was considered significant when it was equal or less than 0.05.

RESULTS

Based on the V2-V3 region of *16S* rRNA gene sequences, figure (1) shows that the prevalence of lactobacilli species it's 144 (57.60%) of the collected samples were colonized by L. *in***.** This was followed by L. *gasseri* 31 (12.40%), L. *Plantarum* 23 (9.20%), L. *suntoryeus* 17 (6.80%), *L. crispatus* 13 (5.20%), L. *rhamnosus* 9 (3.60%), L. *vaginalis* 5 (2.00%), L. *fermentum* 4 (1.60%), L. *helveticus* 2 (0.80%), and L. *johnsonii* 2 (0.80%).

Table 1 showed the association between vaginal bacterial composition and rate of preterm labor. Women with predominantly lactobacilli species were significantly less liable for preterm labor than those women who lack lactobacilli species in their vaginal samples, 10.4% versus 64.2% (P<0.001). To evaluate the risk of preterm labor associated with non-lactobacilli bacterial colonization, Odds ratio was estimated and it was 15.39 (95% confidence interval of 7.91- 29.95). In other words, women who lack lactobacilli are 15 times more liable to have preterm labor than women who have vaginal lactobacilli colonization.

DISCUSSION

The present study shows that pregnant women who lack lactobacilli are 15 times more liable to have preterm labor than women who have vaginal lactobacilli colonization. In accordance with present study it has been estimated that bacterial vaginosis, alteration in the endogenous vaginal microflora with an absent or decreased proportion of lactobacilli and dominance of *G. vaginalis, Prevotella bivia, Mobiluncus sp., Mycoplasma hominis*, and *A. vaginae* (Schwebke *et al.,* 2014), is associated with a 40% increase in the risk of preterm labor (Ugwumadu, 2002). Women with an abnormal vaginal flora in their first 3 months of pregnancy have a higher risk of delivering preterm babies (Van De Wijgert *et al.,* 2014).

Some of these micro-organisms possess sialidase activity, which has been shown to be accompanied by an increased rate of preterm birth (Smayevsky *et al.,* 2001). Sialidases are hydrolytic enzymes that have a role in inhibiting the innate immunity by breaking down the immunoglobin-A (IgA), and because of that action, it has been utilized in many diagnostic materials. Greater LPS amounts, mostly from *P. bivia* (Aroutcheva *et al.,* 2008), and the amount of pro-inflammatory molecules IL-6, IL-1β, and IL-8 have been shown to be increased in the cervical and vaginal fluid of pregnant ladies with BV (Mitchell and Marrazzo, 2014). The increment in vaginal fluid pH higher than 4.5 is a criterion of

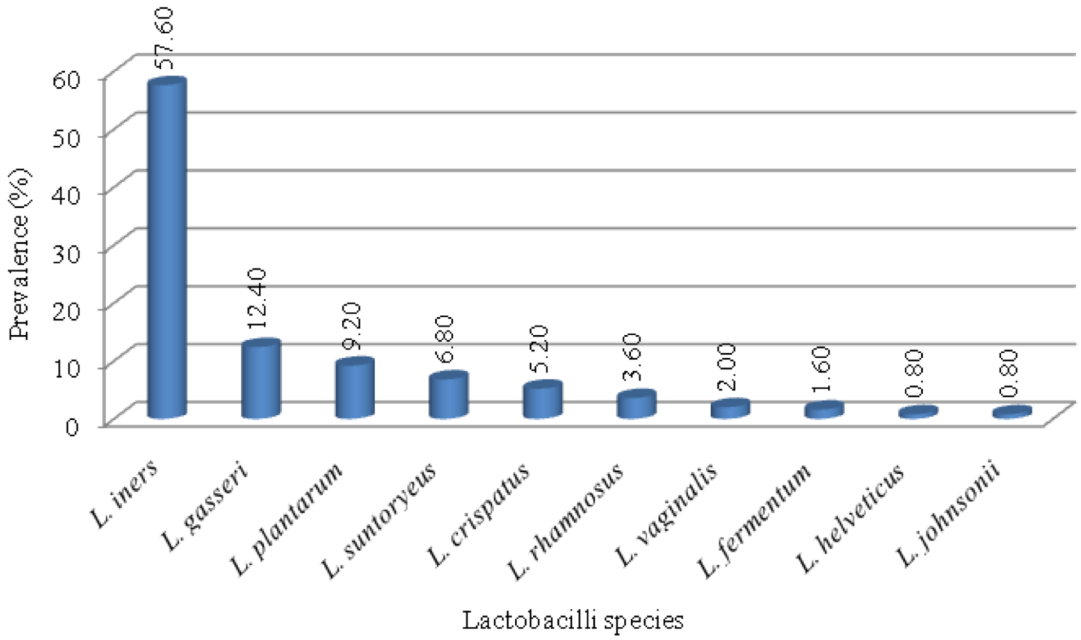

Figure 1: Percentage of women according to Lactobacillus species

Table 1: Association between vaginal bacterial composition and preterm labour

Pregnancy outcome	Pregnant women with no lactobacilli n = 106 n(%)	Pregnant women with predominantly lactobacilli n = 144 n(%)	P-value	Odds ratio	95% CI
PTL	68 (64.2)	15 (10.4)	<0.001	15.39	7.91- 29.95
Term labour	38 (35.8)	129 (89.6)			

N: number of cases; PTL: preterm labour; CI: Confidence interval

BV, and this favors *Lactobacillus iners* on *Lactobacillus crispatus*, because of adaptation via up-regulation of genes responsible for controlling carbohydrate metabolism (Macklaim *et al.,* 2013).

Future researchers must study the activity of these micro-organisms in the uterus and even in the placenta (Aagaard *et al.,* 2014). This may be facilitated by the use of metabolomic analysis to aid disclosing how the vaginal microbiome might affect the PTB risk.

CONCLUSION

In conclusion, the risk of preterm labor is significantly increased with bacterial vaginosis in favor of alteration of vaginal flora with deficient lactobacilli species.

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