Full Length Research Paper

A study of the role of hydrogen peroxide production by lactobacilli in preterm labor

Alaa Mosbah[1]* and Maggie Reda Mesbah[2]

[1]Obstetrics and Gynecology Department, Faculty of Medicine, Mansoura University, Egypt.
[2]Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt.

Some species of lactobacilli are hydrogen peroxide (H_2O_2) producers which may have a protective effect against vaginal colonization by pathogenic species. The aim of this study was to investigate the lactobacillary flora in normal pregnant women and pregnant women with preterm labor, by smear and Nugent score and to assess the distribution of lactobacilli generating hydrogen peroxide in both groups and its correlation to preterm labor. Vaginal specimens were obtained from 60 normal pregnant women and 40 pregnant women with preterm labor with intact membranes. Leukocytic counts, pH detection and Nugent score were done. Isolation and semi-quantification of vaginal lactobacilli on Man-Rogosa-Sharp media (MRS) and then identification of lactobacilli by detection of 1,350 bp fragment of 16S rRNA gene by polymerase chain reaction (PCR) were done. Lactobacilli were finally tested for their production of hydrogen peroxide. Nugent score was significantly higher in women with preterm labor with intact membranes than normal pregnant women (p<0.001) . There was significant high isolation of lactobacilli in normal pregnant women as it was isolated from 47(78.3%) normal pregnant women and from 15(37.5%) pregnant women with preterm labor with intact membranes (p<0.001). As regards hydrogen peroxide production from the isolated strains, there was a highly significant difference between both groups as after 30 min, hydrogen peroxide producing lactobacilli were isolated from 59.6% of normal pregnant women isolates and 20% of preterm labor women isolates (P<0.001). After 1 h, the percentage of isolation had increased to 72.5 and 30% in both groups, respectively. With regard to the pregnancy outcomes, the incidence of preterm delivery was significantly reduced in the strong positive group. Hydrogen peroxide production by vaginal lactobacilli may be used as a simple test for detection of women at high risk of preterm labor.

Key words: Lactobacilli, hydrogen peroxide, vaginal flora, pregnant women.

INTRODUCTION

The composition of vaginal flora is the focus of interest of recent investigations because of its importance to women's reproductive organ and general health (Patta et al., 2008). The vagina is a dynamic ecosystem that is balanced due to the interaction of factors of the native bacterial biota. In healthy adult women, the normal vaginal pH is < 4.5. The predominant species of lactobacilli maintain a low pH through their fermenting activity which protects the area against the invasion of undesirable microorganisms (Pascual et al., 2006).

Lactobacilli in the vagina have been widely accepted as protecting against inflammation from genital pathogens.

Together with the production of lactic acid, lactobacilli form antibacterial complexes including acidolin, lactacin B, and hydrogen peroxide (Cadieux et al., 2002).

Lactobacilli like other lactic acid bacteria lack the heme group and do not utilize the cytochrome system for terminal oxidation. They possess flavoproteins which transform oxygen into hydrogen peroxide. This mechanism together with the absence of the catalase hemoprotein generates H_2O_2 in amounts that exceed the degrading capacity of the organism (Aroucheva et al., 2001).

Hydrogen peroxide alone or in combination with halide and peroxidase that are present in vaginal secretions has potent toxic properties. *In vitro* studies have documented that hydrogen peroxide producing-lactobacilli are cidal to HIV-1, *Gardenerella vaginalis*, bacteriods species *Neisseria gonorrhea* and *Candida albicans*, as H_2O_2

*Corresponding author. E-mail: alaamosbah@hotmail.com.

generates cytotoxic reactive oxygen molecules, super-oxide and hydroxyl radicals in the vaginal fluid (Martinze et al., 2008; Vitali et al., 2007).

Bacteria which colonize the genital tract produce phospholipases that stimulate the release of prostaglandin (Yoon et al., 1995; Onderdonk et al., 2003).

The production and secretion of prostaglandin (PG) has been reported to be important as a mechanism of full term delivery (MacDonald and Casey, 1996).

Preterm delivery generally refers to delivery before 37 weeks of gestation and it continues to be a major obstetric problem as its incidence is increasing throughout the world. The rate of spontaneous preterm delivery has increased by more than 50% in low-risk primiparous women in the last decade (Bennett, 2007).

Preterm delivery occurs in over 7% of all pregnancies, accounts for 75% of neonatal deaths, and causes significant neonatal morbidity (Challis, 2000).

Various etiologies may interact to result in early effacement and dilatation and subsequent spontaneous preterm delivery. They include cervical weakness, infection (ascending, haematogenous or iatrogenic), decidual hemorrhage and placental abruption, uterine distension secondary to multiple pregnancy, maternal illness (e.g. intercurrent pyelonephritis) and fetal stress (e.g. polyhydramnios secondary to gastrointestinal atresia, twin-to-twin transfusion syndrome) (Bennett, 2007).

However, the main cause of preterm delivery is premature rupture of the membranes (PROM) with incidence ranging from 3 -18.5%. The greatest risk factor for PROM is infection as bacterial proteases decrease the strength and elasticity of the chorioamniotic membranes (Parry and Strauss, 1998).

The mechanism of preterm labor is believed to be different from that of full term labour and it has been reported that it may be due to the activation of cell mediated immunity in response to bacteria or bacterial products (Hitti et al., 2001).

In particular cell mediated immunity initiated by infection in the uterus, induces the production and secretion of various cytokines (IL-1, IL-6, IL-8, tumor necrosis factor) and PG. These proinflammatory cytokines cause weakening of the fetal membranes by disrupting the extracellular matrix and releasing enzymes such as metalloproteinase which increase collagen degradation (Usui et al., 2002; Money, 2005).

It was thought that ascending bacterial infection in the vagina through the uterine cervix may play a more significant role than hematological infection (Kim et al., 2006). Many recent studies have evaluated the lactobacillary flora and its protective role against vaginal infections but there is still controversial data.

Aim of the work

The aim of this study was to investigate the lactobacillary flora in normal pregnant women and pregnant women with preterm labor with intact membranes, to compare vaginal flora by smear and scores by Nugent criteria on Gram stain and to assess the distribution of lactobacilli generating-hydrogen peroxide in both groups and its correlation to preterm labor.

SUBJECTS, MATERIALS AND METHODS

Subjects

This study was performed on 100 pregnant women in the Gynecology and Obstetric Department in Mansoura University Hospital (MUH) from March 2008 to October 2008. The study group comprises 40 pregnant women with preterm labor whose gestational age ranged from 28 to 37 weeks of gestation and 60 normal pregnant women with gestational age above 37 weeks. An informed consent was signed by every woman and then the following were done for each case:

Determination of gestational age

Gestational age was determined by using the last menstrual period (LMP) and earliest ultrasound. If the ages determined by using LMP and earliest ultrasound were concordant within the defined limits of ultrasound (within 7 days of each other for the gestational period ranging from 0 - 12 weeks; within 10 days of each other for gestational period ranging from 12 - 20 weeks; within 2 weeks of each other for a gestational period ranging from 24 - 28 weeks; and within 3 weeks of each other for a gestational period of longer than 28 weeks), the gestation age determined by using the LMP was taken to be correct. If the results were discordant the age determined by using the earliest ultrasound was taken to be correct.

Collection of samples for microbiologic analysis (Nugent et al., 1991; Wilks et al., 2004)

All the subjects underwent speculum examination and from each of them, three vaginal samples were collected with cotton tipped swabs distal to the speculum from the posterior zone of the fornix of the vagina. The first swab was used to make saline wet mount and for pH detection. The second swab was used to make Gram-stained film for Nugent scoring. The third swab was placed in 2 ml of 0.1 M Tris-HCL buffer, pH 7.4 and vortexed for 30 s to disperse adherent bacteria into the buffer solution. Two hundred microlitres of the buffer solution was inoculated on an anaerobic cryogenic pre-servative pre-reduced brain heart infusion broth (10% glycerol and 0.002 resazurin) and stored at -70°C for subsequent microbiological analysis.

Wet smears of vaginal discharge and pH detection (Kim et al., 2006)

The first swab was rolled onto a glass slide with saline. pH was measured using an indicator strip (pH 1.0 - 14.0 universal indicator Merck, Germany) and the number of leukocytes was counted using a microscope under ×40 magnification.

Gram stain (Nugent et al., 1991)

The second swab sample was used to prepare dried and fixed film, stained by Gram stain and were scored according to the Nugent score. This method scores the smears in a standardized quantification of some of the cell types present, which were designated

Table 1. Nugent scoring system (0-10) for Gram-stained vaginal smear.

Score	*Lactobacilli* morphotype (Long bacilli)	*Gardenerella* and *Bacteriods* sp. (Short bacilli)	Curved Gram variable rods (*Mobiluncus*)
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

Morphotypes are scored as the average number seen per oil immersion field: 0, no morphotype present; 1, 1 morphotype present; 2, 1 to 4 morphotypes present; 3, 5 to 30 morphotypes present; 4, 30 or more morphotypes present.

as lactobacilli morphotype (long bacilli), *Gardenerella* and *Bacteroids* species morphotype (short bacilli) and curved Gram variable rods, that is *Mobiluncus* as shown in Table 1.

The scoring system 0 to 10 uses the quantitation (0, 1 to 4+) of the following morphotypes to yield a total score of 0 to 10 per person of the three morphotypes: large Gram positive rods (lactobacillus morphotype) whose absence yielded a high score, small Gram-negative to variable rods (*G. vaginalis* and *Bacteroids* sp. morphotypes) and curved Gram variable rods (*Mobiluncus* sp. morphotype). A score of zero to three is considered to be normal. Four to six is considered intermediate and seven to ten is defined as bacterial vaginosis.

Isolation, semi-quantitative culture and preliminary identification of lactobacilli species (Wilks et al., 2004; Mijac et al., 2006)

Lactobacilli were recovered from frozen samples by inoculation of serial 10-fold dilutions in phosphate buffer saline on an MRS agar (Difco, Detroit, MI, USA) which was incubated in an anaerobic jar with an anaerobic gas generating kit (Anaerogen/Campygen gas pack 80% N_2; 10% CO_2; 10% H_2) Oxoid for 48 h at 35°C. Serial dilutions were used to allow identification of distinct colonial types.

Preliminary identification of lactobacilli was performed by colony morphology, Gram stain, and catalase and oxidase tests. Gram positive long bacilli which were negative for both catalase and oxidase tests were identified preliminarily as lactobacilli.

Single colonies were sub- cultured onto an MRS agar and incubated anaerobically at 35°C for 48 h. These cultures were used for DNA extraction and for the determination of H_2O_2 production.

Identification of lactobacilli (Wilks et al., 2004)

Lactobacilli were identified by amplification of 1,350-bp fragment of 16S rRNA using oligonucleotide primer targeted at highly conserved regions of bacterial 16S rRNA genes.

DNA isolation

QIA amp DNA minikit (QIAGEN Inc., Valencia, CA, USA) was used. 1 ml of overnight bacterial growth on MRS broth was centrifuged at 3000 rpm for 3 min. 200 µl of the deposit was put into a microfuge tube, 180 µl buffer ATL was added and 20 µl proteinase K was added and mixed by votex. After incubation at 56°C for 1 h, 200 µl of the buffer AL was added and mixed by pulse-vortexing for 15 s and incubated at 70°C for 10 min. 200 µl of ethanol (100%) was added to the sample and mixed by pulse vortexing for 15 s and the mixture was applied to QIA amp spin column and centrifuged at 8000 rpm for 1 min. 500 µl buffer AW1 was added to the QIA amp

spin column and centrifuged as before.

500 µl buffer AW2 was added to the QIA amp spin column and centrifuged at 14000 rpm for 3 min. 200 µl distilled water was added to the QIA amp spin column and incubated at room temperature for 5 min, and then centrifuged at 8000 rpm for 1 min; the elute was collected in sterile tubes and stored at -20°C until PCR reaction was done.

PCR

Primer

Synthetic oligonucleotide primers for a 1, 350- bp fragment of 16S rRNA gene - GAA CGC TGG CGG CGT GCC (Z1-forward) and - TCC GCG ATT ACT AGC GAT TCC (Z2- reverse) (Promega) were targeted at highly conserved regions of the bacterial 16S rRNA genes (Promega) (Wilks et al., 2004).

PCR protocol

The PCR reaction was done in total volume of 50 µl: two µl of DNA, 1 µl of sense primer and 1 µl of antisense primer were added to 46 µl of PCR master mix DyNAzymeII (Finnzymes, Espoo, Finland). This provides 1U of enzyme, 1.5 mM $MgCl_2$ and 200 µM dNTP in the final reaction concentration. The thermal cycle program was adjusted as follows: initial denaturation step for 4 min at 94°C; three step, cycling ×35 times; denaturation step, 30 s at 94°C; annealing step, 30 s at 45°C; extension step, 60 s at 72°C; final extension, 10 min at 72°C. Agarose gel electrophoresis of the amplified DNA with DNA standard marker was: X174/Hae digest marker (Promega) that detected the 1,350- bp band

Detection of hydrogen peroxide production (Kim et al., 2006; Mijac et al., 2006)

At least, 3 colonies of each isolated and identified strain by PCR were inoculated in MRS liquid media supplemented with 30% glycerol and stored at -70°C until used. For H_2O_2 detection, 3 µl from each isolated strain was plated on the MRS agar containing 0.25 mg/ml tetramethyl-benzidine and 0.1 mg/ml of horseradish peroxidase (Sigma, St Louis MO, USA) and incubated for 5 days at 37°C under anaerobic conditions. After exposure to ambient air, hydrogen peroxide producing colonies turned blue in 20 min. The colony count was assessed after 30 min and 1 h. Colonies showing a blue colour were scored as hydrogen peroxide positive (+).

According to the intensity of the colour, Prussian blue was scored as strongly positive, blue was positive, and light blue was weakly positive and unchanged colour was scored as negative.

Table 2. pH and WBC count in normal pregnant women and women with preterm labor with intact membrane.

Parameter	Normal pregnancy n= 60	Preterm labor n= 40	p- value
PH	5.18 ± 1.6	5.01 ± 1.3	p<0.09
WBC	4.88 ± 2.6	7.3 ± 2.9	p<0.001***

Values represent means ± S.D. p<0.001***.

Table 3. Gram stain results from vaginal fluid of normal pregnant women, and women with preterm labor with intact membranes.

	Normal pregnancy n= 60	Preterm labor n= 40	p -value
Nugent score	1.28 ± 1	2.4± 0.8	P<0.001***
Lactobacillus	2.7 ± 1.1	1.5 ± 0.74	P<0.001***
Bacteriods and Gardenerella	1.2 ± 0.85	2.6 ± 1.17	P<0.001***
Mobiluncus	2.03 ± 1.14	2.15± 1.14	P<0.62

Values represent means± S.D. p<0.001***.

Statistical analyses (Munor et al., 2002)

The statistical analyses of data were done by using excel and SPSS (statistical package for social science version 10) programs. The description of the data was done in form of mean (+/-) SD for quantitative data, frequency and proportion for qualitative data. The analysis of the data was done to test statistical significant differences between groups. For the quantitative data, student t - test was used to compare between the 2 groups. Chi-square test was used to compare qualitative data.

P is significant if < or = 0.05 at confidence interval of 95%.

RESULTS

Wet smear and pH of vaginal discharge

The number of leukocytes in the vaginal discharge of preterm-labor women without ruptured membranes was 7.3 ± 2.9, which was significantly higher than that of normal pregnant women, which was 4.88 ± 2.6 (p < 0.001***). The pH value of vaginal discharge in preterm labor women was 5.01 ± 1.3 and that of normal pregnant women was 5.18 ± 1.6; a significant difference between the two groups was not detected (Table 2).

Gram stain of vaginal discharge and Nugent score

As detected by Gram staining of vaginal discharge, lacto-bacilli morphotype in preterm labor women was 1.5 ± 0.74 which was significantly lower than in normal preg-nant women which was 2.7 ± 1.1(p < 0.001). *Gardnerella* and *Bacteriods* morphotype was 2.6 ± 1.17 in preterm labor women, and was significantly higher than normally found 1.2±0.85 (p<0.001***). *Mobiluncus* organisms were 2.15±1.14 and 2.03±1.14, respectively and a significant difference between the two groups was not detected (p < 0.62). The Nugent score in preterm labor women was 2.4

± 0.8 which was significantly higher than in normal pregnant women 1.28 ± 1 (p < 0.001***) (Table 3).

Isolation of lactobacilli

From 60 normal pregnant and 40 pregnant women with preterm labour having intact membranes, all Gram positive bacilli which were oxidase and catalase negative were identified by PCR (Figure 1). The percentage of the isolation of lactobacilli in these pregnant women (both normal and preterm labour) is shown in Table 4. There was a high significant isolation of lactobacilli from normal pregnant women (47 isolate, 78.3%), than pregnant women with preterm labor having intact membranes (15 isolate, 37.5%; p < 0.001***).

Hydrogen peroxide production

In normal pregnant women, 130 colonies of vaginal lactobacilli were cultured and isolated from 47 identified strains, and in preterm labor women with intact membranes, 40 colonies were cultured and isolated from 15 identified strains.

Hydrogen peroxide production in the above mentioned strains was detected as follows: after 30 min, 20% of the colonies was positive in preterm labor women with intact membranes which was significantly lower than the normal pregnant isolates in which 59.6% of the colonies was positive (p < 0.05). Also, there was a significant difference between both groups as regard the strong positive, weak positive and negative groups (Table 5).

After 1 h, the number of hydrogen peroxide-producing lactobacilli colonies rose to 30 and 72.5% in both groups, respectively (p < 0.05). Also, there was a significant

Table 4. Percentage of isolation of *Lactobacilli* (LB) from normal pregnant women and women with preterm labor with intact membranes.

	Normal pregnancy n= 60		Preterm labor n= 40		p value
	No.	%	No.	%	
With LB	47	78.3%	15	37.5%	p<0.001***
Without LB	13	21.7%	25	62.5%	p<0.001***

p<0.001***.

Table 5. Hydrogen peroxide producing lactobacilli from vaginal fluid of normal pregnant women and women with preterm labor with intact membranes after 30 min.

	After 30 min				
H_2O_2 production	Normal pregnant (n= 47)		Preterm labour (n=15)		p -test
	n	%	n	%	
Strong positive	25	19.2	2	5	0.03*
Positive	14	10.6	6	15	0.46
Weak positive	38	29.8	0	0	<0.001***
Negative	53	40.4	32	80	<0.001***
Total colony count	130	100	40	100	

p < 0.05*; p < 0.001***.

Table 6. Hydrogen peroxide producing lactobacilli from vaginal fluid of normal pregnant women and women with preterm labor with intact membranes after 1 h.

	After 1 h				
H_2O_2 production	Normal pregnancy		Preterm labour		p -test
	n	%	n	%	
Strong positive	44	33.5	1	2.5	<0.001***
Positive	14	11.5	6	15	0.46
Weak positive	36	27.5	5	12.5	0.04*
Negative	36	27.5	28	70	<0.001***
Total	130	100	40	100	

p<0.05*; P<0.001***.

Table 7. Pregnancy outcomes of women who were positive for hydrogen peroxide-producing lactobacilli after 1 h of incubation.

Parameter	Positive group (n= 18)	Negative group (n= 20)	P value
Gestational age (weeks)	37.34 ± 3.02	32.31± 2.09	P<0.001***
Preterm delivery(<37weeks)	2 (11%)	8 (40%)	P=0.04*

p<0.05*; p <0.001***.

difference between both groups as regard the strong positive, weak positive and negative groups (Table 6).

Pregnancy outcomes of women who were positive for hydrogen peroxide producing lactobacilli

The pregnancy outcomes between the two groups showing positive or negative response for H_2O_2 production after 1 h incubation were compared (Table 7). Patients who had equal number of positive and negative hydrogen peroxide-producing colonies were excluded to minimize the confusion. The gestational age was significantly higher in the positive group than in the negative group (p < 0.001***). Preterm delivery rate was significantly higher
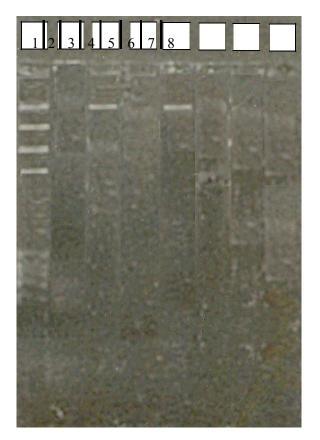
Figure 1. The agarose gel (1.5%) electrophoresis stained by ethidium bromide. Lane 1 represents X174/Hae DNA marker; Lanes 3, 5 show 1, 350-bp bands of 16s rRNA gene; Lanes 2, 4, 6, 7 and 8 are negative.

in the negative group than in the positive group (P= 0.04*).

DISCUSSION

Preterm birth occurs in over 7% of all pregnancies, accounts for 75% of neonatal deaths, and causes significant neonatal morbidity (Challis, 2000).

Various etiologies may interact to result in early cervical effacement and dilatation and subsequent spontaneous PTB. However, the cause of preterm labour cannot be identified in many cases. Many reports have been evidenced that a substantial portion of idiopathic preterm labour is related to intrauterine infection (Bennett, 2007).

Lactobacilli present in the vaginal flora decreases vaginal pH by producing lactic acid and thus mediate the first line of protection against vaginal infection. In addition, hydrogen peroxide production by some lactobacillus species has been found to suppress the growth of many microbial organisms (Dickson et al., 2006).

The intention of our study was to investigate the hydrogen peroxide producing lactobacilli and its association with preterm labor by comparing its presence in pregnant women with preterm labour having intact membranes and normal pregnant women and to study its association with the outcome of pregnancy.

In our study, wet smears of vaginal discharge, pH measurement, Gram staining and Nugent score have been done with results obtained. Then, lactobacilli were isolated on an MRS media and Gram positive bacilli which were catalase and oxidase negative were identified by detection of 16s rRNA gene by PCR. Hydrogen peroxide production by lactobacilli was tested on an MRS media containing 0.25 mg/ml tetramethyl-benzidine and 0.1 mg/ml of horseradish peroxidase.

With regards to the number of leukocytes in the vaginal smear test, it was significantly higher in preterm labor women with intact membranes (7.3 ± 2.9) than in normal pregnant women (4.88 ± 2.6; $p < 0.001^{***}$). pH detection showed no significant difference between the two groups ($p < 0.09$).

These results were in accordance with Kim et al. (2006) who studied the hydrogen peroxide- producing lactobacilli in the vaginal flora of pregnant women with preterm labor having intact membranes, and they detected that the number of leukocytes in preterm labor women without rupture of membranes was significantly higher than in normal pregnant women which reflects inflammation in the vaginal smear test.

With regard to the Gram stain, the Nugent score was found to be significantly higher in preterm labor with intact membranes whose score was 2.4 ± 0.8 than in normal pregnant women (1.28 ± 10; $p < 0.001$). The lactobacillus morphotype was significantly higher in normal pregnant smears (2.7 ± 1.1) while the *Bacteroids* and *Gardenerella* morphotype were significantly higher in preterm labor group (2.6 ± 1.17; $p < 0.001$) . Our results are in parallel to Kim et al. (2006) whose Nugent score result was significantly higher in preterm labor (2.34 ± 0.51) than in normal pregnant women (1.21 ± 0.30). This high score means that bacterial vaginosis is higher in pregnant women with preterm labor with intact membranes than normal pregnant women and that there is a reduction of their lactobacillary morphotypes. This is documented to be associated with serious pregnancy complications including premature rupture of membranes, preterm delivery and postpartum endometritis (Martinze et al., 2008).

Also, Vitali et al. (2007) who studied the dynamics of bacterial flora in normal women and those with bacterial vaginosis found that there was marked reduction in hydrogen peroxide producing lactobacilli prior to developing bacterial vaginosis.

It is accepted that the Nugent scoring system yielded an improvement in inter- centre agreement of evaluation of vaginal flora (Verhelst et al., 2005). Also, Fredricks et al. (2007) detected 96% sensitivity and 94% specificity for Nugent score.

Controversially, Core et al. (2002) who studied the Gram stain diagnosis of bacterial vaginosis after rupture

of membranes found that it had a good negative predictive value (63%) but it had a poor sensitivity.

Lactobacilli were isolated and identified by the detection of 16s rRNA gene by PCR. There was a significant higher detection of lactobacilli in normal pregnant women (47, 78.3%) versus pregnant women with preterm labor with intact membranes as it was isolated from 15 (37.5%) women ($p < 0.001$). Similarly, Wilks et al. (2004) who identified vaginal lactobacilli and studied its H_2O_2 production in women at high risk of preterm birth documented 16s rRNA based methods as a reliable method for the identification of lactobacilli.

With regard to hydrogen peroxide production from the isolated strains of lactobacilli, it was found that in women with preterm labor, lactobacilli producing-hydrogen peroxide as a normal host response was significantly reduced after 30 min as it was detected in 20% of preterm labor women versus 59.6% in normal pregnant women. However, after 1 h the detection rate had increased to 30 and 72.5% in both groups, respectively ($p < 0.05$).

Our results are in accordance with Kim et al. (2006) who detected significant reduction of H_2O_2 producing lactobacilli in preterm labor women with intact membranes as it was detected in 16.9 and 40.3% after 30 min and 1 h respectively. Also, Mijac et al. (2006) who studied hydrogen peroxide producing lactobacilli in women with vaginal infections reported a significant lower colonization with lactobacilli in bacterial vaginosis than in the control group.

Supportive to our results, Wilks et al. (2004) found that the presence of lactobacilli producing high level of H_2O_2 in the vagina of pregnant women was associated with reduced risk of bacterial vaginosis, chorioamnionitis and preterm birth. These results mean that H_2O_2 producing lactobacilli which are considered a normal host defense mechanism were significantly reduced in the case of preterm labor. This lower detection may imply a change of the vaginal flora due to pregnancy, although other factors may also cause a change in vaginal flora. The high Nugent score together with a lower detection of the H_2O_2-producing ability of lactobacilli is thought to play a significant role in protecting the vaginal ecosystem from bacterial vaginosis infection, although direct evidence to support this notion is lacking

Our results of the pregnancy outcome among females whose isolated lactobacilli were H_2O_2 producers and those with negative H_2O_2 production showed that the positive group had a significant higher gestational age at delivery (37.34 ± 3.02; $p < 0.001$)) and significant lower incidence of preterm labor as it occurred in 2 cases (11%; p= 0.04) which were different from Kim et al. (2006) who detected no difference in the outcome of pregnancy in both groups.

Tests that assess various microorganisms inducing preterm labor are time consuming and therefore expensive. In this study, lactobacilli generating hydrogen peroxide that mediate the host defense system in the vagina were measured and it was found that their distribution was decreased significantly in the preterm labor group. So, we postulate that H_2O_2-producing lactobacilli are able to reduce the incidence of ascending infections of the uterus which is the most important cause of PTB and in the future, this test may be applied usefully for the early detection and thus prevention and treatment of preterm labor.

Our results suggest that tests for determining the presence of vaginal H_2O_2-producing lactobacilli may be a clinically useful tool for identifying women at an increased risk of preterm delivery.

REFERENCES

Patta MC, Nunes SA, Moreira JL, Anukam KC, De Mrtinis GR (2008). Analysis of vaginal lactobacilli from healthy and infected women. Appl. Environ. Microbiol. 14: 4539-454.

Pascual LM, Daniel MB, Pajaro C, Barberis L (2006). Lactobacillus species isolated from vagina: Identification, hydrogen peroxide production and non oxynol -9 resistance. Contracep. 73: 73-81.

Cadieux P, Burton J, Gardiner G, Braunstein J, Bruce AW, Kang GY, Reid G (2002). Lactobacilli strains and vaginal ecology. J. Am. Med. Assoc. 287: 1940-1941.

Aroucheva A, Gariti D, Simons M (2001). Defense factors of vaginal Lactobacilli . Am. J. Obstet. Gynecol. 185: 375-379.

Martinze RCR, Franceschini SA, Patta MC, Nunes, AC, Moreira, JLS, Anukam KC, Martinis De RCP (2008). Analysis of vaginal lactobacilli from healthy and infected women. Appl. Environ. Microbiol. 74(14): 4539-4542.

Vitali B, Pugliese C, Biagi E, Candela M, Ballen ST, Donders GG, Brigidi P (2007). Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis ,or no infection ,analyzed by denaturing gradient gel electrophoresis and real time PCR. Appl. Environ. Microbiol. 73(18): 5731-5741.

Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH (1995). Amniotic fluid interleukin-6: A sensitivity test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. Am. J. Obstet. Gynecol. 172: 960-970.

Onderdonk AB, Lee ML, Liberman E, Delaney MT, Tuomala T (2003). Quantitative Microbiologic Models for preterm delivery. J. Clin. Microbiol. 41(3): 1073-1079.

MacDonald PC, Casey ML (1996). Preterm birth. Sci. Am. 3:42-52.

Bennett P (2007). Preterm labor In: Edmonds DK ed. Dewhurst textbook of obstetrics and gynecology. 7th ed. London: Blackwell pp.177-191.

Challis JRG (2000). Mechanism of parturition and preterm labor. Obstet. Gynecol. Surv. 55: 650- 660.

Parry S, Strauss JF (1998). Premature rupture of the fetal membranes. N. Engl. J. Med .338: 663-670.

Hitti J, Hillier SL, Agnew KJ, Krohn MA, Reisner DP, Eschenbach DA (2001). Vaginal indicators of amniotic fluid infection in preterm labor .Obst. Gynecol. 97:211-218.

Usui R, Ohkuchi A, Masubara S, Lzumi A, Watanabe T, Suzuki M (2002). Vaginal lactobacilli and preterm birth. J. Perinat. Med. 30: 548-66.

Money D (2005). The laboratory diagnosis of bacterial vaginosis. Can J. Infect. Dis. 6(2): 27-79.

Kim YH, Kim CH, Cho MK, Na JH, Song TB, OH JS (2006). Hydrogen peroxide producing lactobacilli in the vaginal flora of pregnant women with preterm labor with intact membranes. Inter. J. Gynecol. Obstet. 93: 22-27.

Nugent RP, Krohn MA, Hillier SL (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J. Clin. Microbiol. 29: 297-301.

Wilks M, Wiggins R, Whiley A, Hennessy E, Porter HS, Corfieled A, Millar (2004). Identification and H2O2 production of vaginal lactobacilli from pregnant women at high risk of preterm birth and relation with outcome of pregnancy. J. Clin. Microbiol. 42(2): 713-717

Mijac VD, Dukic SV, Opavski NZ, Dukic MK, Ranin LT (2006). Hydrogen peroxide producing lactobacilli in women with vaginal infections. Sci. Direct. Euro. J. Obstet. Gynecol. Reprod. Biol. 129: 69-76.

Munor BH, Jacobsen BS, Duffy ME, Bratmer LE (2002). Statistical methods for health care research .4thed. Lipincott. USA. pp. 1-412.

Dickson EM, Rggio MP, Macpherson L (2006). A novel species – specific PCR assay for identifying lactobacillus permentum. J. Med. Microbiol. pp. 299-303.

Verhelst R, Verstraelen H, Glaeys G, Verschraegen VS, De Ganck, C, Backer ED, Temmerm M, Vaneechoutte M (2005). Comparison between Gram stain types that resemble grade I microflora and revised categorization of grade I microflora. BMC. Microbiol. 5: 61-69

Fredricks DN, Fiedler TL, Thomas KL, Ookley BB, Marrazzo JM (2007). Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. J. Clin. Microbiol. 45(10).

Core LA, Mastrobattista JM, Bishop K, Newton ER (2002). Gram stain diagnosis of Bacterial vaginosis after rupture of membranes. Am. J. Perinatol. 17: 315-358.