Association between Lactobacillus species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in small population of pregnant Latvian women

Natalija Berza [1,2*], Jana Zodzika [2], Juta Kroica [1], Aigars Reinis [1], Ingus Skadins [1], Linda Piekuse [1], Polina Zalizko [1], Olita Melngaile [2], Rita Pundure [2], Irina Lukojanova [2], Olga Vasina [2]

[1]Department of Biology and Microbiology, Riga Stradins University, Dzirciema Street 16, Riga, Latvia
[2] Department of Gynecology and Obstetrics of Riga Stradins University, Dzirciema Street 16, Riga, Latvia

**** Corresponding Author:*** Natalija Berza
Address: Lokomotives Street 70a-27, Riga, LV-1057, Latvia
Email: natalija.berza@gmail.com

Abstract

Background: One of the most common reasons why females attend doctor are vaginal infections. Vaginal flora is a dynamic environment where a great variety of microorganisms exist in homeostasis. The main normal flora inhabitants are *Lactobacillus* species who protect from pathogens. Still the majority of factors remain unclear about this genteel environment and its interaction.

Aim & Objectives: to analyze vaginal microflora types and microbial species in pregnant women, who were at their first trimester, using PCR and *Nugent* score diagnostic methods.

Methods: 65 pregnant women before their 12th week of pregnancy participated in this study from 06.08.2012 till 31.01.2013. All participants were divided in to 2 groups, group A (n=45) normal pH and group B (n=20) pH (≥4,5). Their vaginal fluid were analysed with *Nugent* score and PCR methods.

Results: Genus *Lactobacillus* (any *Lactobacillus*) was detected by PCR in all women irrespective of Nugent score, most common species were *L. crispatus*, *L. jensenii*, and *L. inners,* less common were *L. gasseri, L. plantaris*, *L. rhamnosus* and *L. reuteri.*
G. vaginalis was present in both patient groups divided by pH but it was significantly higher in bacterial vaginosis and intermediate flora group detected by *Nugent* score and group B (high pH group). *Megasphaera*, *Leptotrichia/Sneathia* were detected more common in pathogenic flora than normal flora. *A. vaginae* finding was associated with pathologic flora.

Conclusion: The most common isolated species in pregnant women vaginal flora were *L. crispatus*, *L. jensenii*, and *L. inners. L. gasseri* and *L. plantaris* were second most often found

species. *L. jensenii* detection was significantly higher in normal flora group. *A. vaginae* were mainly detected in patients with bacterial vaginosis. *Megasphera* and *Leptotrichia/Sneathia* are more common for patients with pathologic flora. PCR method is the most precise to identify microorganisms in vaginal flora but rather expensive and time consuming than vaginal fluid examination by *Nugent* score.

Key words: Vaginal microflora, PCR, *Lactobacillus*, *Nugent* score.

Introduction

One of the most common gynaecological problems is vaginal infections like bacterial vaginosis (BV), *Candida* vulvovaginitis, trichomonal infection and aerobic vaginitis (AV). BV is caused by an overgrowth of *Gardnerella vaginalis (G.vaginalis)*, anaerobes, *Mycoplasma hominis (M.hominis), Ureaplasma urealyticum (U.urealyticum)* and clinically diagnosed by *Amsel* criteria: presence of thin, grayish homogenous discharge; vaginal pH greater than 4.5; presence of clue cells, positive whiff test (detection/enhancement of fishy odor on additions of potassium hydroxide to the vaginal specimen). AV is absence of lactobacilli, presence of cocci or coarse bacilli, parabasal epithelial cells, vaginal leucocytes. AV is associated with growth of group B streptococci (GBS), *E.coli, Staphylococcus aureus (S.aureus)*, has different immunological inflammation reaction and clinical signs – such as red, inflamed vaginal mucosa, yellowish sticky discharge, high vaginal pH, "not fish-like" odor.[1,2] Vaginal infection especially bacterial vaginosis and aerobic vaginitis can be the reason for such complications as preterm delivery, horioamnionitis and low birth weight. The studies with antibiotic treatment in high risk preterm delivery pregnancies have not proven to be effective except clindamycin, but more studies are required.[3,4]

Mostly these infections are asymptomatic, 60% of patients that have bacteria vaginosis (BV) had no complains.[5] It is important to discover all cases of vaginal infections early, because such deviations before 14th week of delivery can be as risk factor for preterm delivery.[3]

There are different diagnostic methods for vaginal infections – pH measure, KOH test, visual vaginal discharge evaluation, dark field microscopy, Grams staining microscopy, cultivation on artificial media and polymerase chain reaction (PCR). There is still a great dilemma - which method is easier, cheaper and more widely available for the specialist. Vaginal pH level is important diagnostic criteria which can be helpful in asymptomatic cases of BV but how precise this method is comparing to other diagnostic methods? [3]

Nowadays, molecular diagnostic methods are becoming more popular, because they provide precise information about species of normal and pathogenic vaginal flora inhabitants. It is not fully understood how microorganisms determined with molecular methods correlate with basic daily tests like pH and *Nugent* score.[6]

Studies show that vaginal microbial composition is dependent on a variety of factors, including geographical, for example, the normal female flora in African women is considered as pathogenic for other region women.[7-10] The most common lactobacillus in India is considered *L.reuteri*, but in Finland *L.crispatus*.[11,12] There were no study data about which Lactobacillus and pathogenic microflora species present in the vaginal flora of Latvian women. So it was quite important to maintain this information and compare it with world data and also find the correlation between molecular diagnostic methods and *Nugent* score.[13]

The goal of this study was to analyze different vaginal flora types by Grams staining and PCR in pregnant women during their first trimester of pregnancy with a special emphasis on lactobacilli and pathogenic bacteria species connected with bacterial vaginosis.

Methods

A total of 65 pregnant Latvian women were enrolled in this study during routine prenatal visits at SIA "Dzirciema Clinic" Ltd "Aura R" and the "Jugla Medical Center" from August 2012 to February 2013. Informed consent was obtained from all participants in verbal and written form. All necessary approvals were received from Riga Stradins University ethical comity. Including criteria in the study were pregnant women older than 18 years with no serious extragenital abnormalities in their 6-12 week of pregnancy. Estimated date of delivery was determined from the last menstrual period and early gestational fetal ultrasonographic measurements. Patients were divided into 2 groups: group A with normal pH (<4,5) and group B with elevated pH (≥4,5), pH set to Machery Nagel pH strips (measuring range 3.6-7.0). A sterile speculum was inserted into the vagina and two specimens of vaginal fluid were obtained by brushing the posterior vaginal fornix with a swab. A vaginal smear was prepared by rolling a swab onto a glass slide, which was then air-dried, heat-fixed, and Gram-stained. The smears were then assessed according to Nugent criteria (Table 3). *Nugent* score for the diagnosis of BV is ≥ 7 and it is considered as pathogenic flora, intermediate flora 4-6 and for normal flora ≤ 3. Overall, the *Nugent* scoring system for Gram –stained vaginal smears has shown high intracenter and intercenter reliability and reproducibility, however practitioners are not usually familiar with performing in-office Gram-stain-based diagnosis, *Nugent*'s criteria are widely applied in the absence of standardized pre-analytical and analytical conditions and interpretation, especially of the so called intermediate flora, is also a matter of concern.[1,6,14] Second specimen was placed in Amies media and immediately transported for molecular investigation.

Swab from Amies media was placed 2 ml containers and the pellet was digested with proteinase K at 56°C for 60–90 min and the DNA was extracted and purified with a QIAmp DNA Investigator Kit (Qiagen, Germantown, MD) in accordance with the manufacturer's instructions, resulting in 100 μl of DNA solution. PCR mixtures consisted of PCR buffer with 1.5 mM of $MgCl_2$, 10 pmol of each primer, 2.0 μM of each deoxyribonucleoside triphosphate, 0.1 μl of *Taq* DNA polymerase, and 1 μl of template DNA solution in a final volume of 25 μl.

Sequences and annealing temperatures for the various primer sets are listed in 1st table. All primers were located in the 16S rDNA region. PCR was carried out for 40 cycles. For the

Lactobacillus genus and its four species, the denaturation was performed at 95°C for 15 sec followed by a 1-min annealing and extension step. For four BV-related bacteria, the denaturation step was set at 94°C for 30 sec, followed by the annealing step for 40 s, with extension at 72°C for 1 min for all reactions. A final extension step at 72°C for 7 min was added for all reactions. Aliquots of 8 μl of the PCR products were electrophoreses in agarose gels and visualized by ultraviolet transillumination after ethidium bromide staining.

The statistical analysis was made using SPSS, Chi square test and Pearson correlation was performed.

Results

There were 65 pregnant women included in the study 45 with pH < 4,5 and 20 with pH ≥ 4,5. The mean age in both groups were 28 ± 5.2. Minimal age of participant was 18 and maximal– 43.

Comparing both pH and *Nugent* score diagnostic methods statistically significant difference was found (X^2= 6,607; p=0,01), high pH was measured only in 17% of participants, but Nugent score shoed pathogenic flora in 37% of participants, (diagram 1).

Based on our data pH test sensitivity compared with Grams staining method was 54% and specificity 88%.

Genus *Lactobacillus* (any *Lactobacillus*) was detected by PCR in all women irrespective of Nugent score, the most common species were *L. crispatus*, *L. jensenii*, and *L. inners,* less common were *L. gasseri, L. plantaris*, but least likely was *L. rhamnosus* and *L. reuteri* species which were found only in two patients specimen (diagram 2).

L. jensenii was detected in normal vaginal flora significantly more frequently *(p<0,01)* than in pathogenic flora, but in *L. inners* detection frequency was no significant difference both in normal and pathogenic flora.

Gardnerella vaginalis was detected in both normal and pathogenic group, but in BV and middle flora by *Nugent* score and high pH group it was detected more often (85-90% of cases) compared with normal pH and 0-3 *Nugent* scores (67-73%). *Megasphaera*, *Leptotrichia* were found less frequent in 0-3 *Nugent s*cores, but *Leptotrichia* detection in both pH groups did not differ statistically. An *A. vagina* was detected mainly in pathogenic flora group.

The incidence of microorganisms, detected with PCR, in vaginal flora, depending on the diagnostic method are displayed in 2nd table.

Performing Pearson correlation test on all summarised data some significant correlations was discovered. The correlation between *Nugent* and pH test was moderate (r=0,453; p<0,01) also there was found moderate correlation between presence of *Megasphera spp.* with *Leptotrichia*

spp. and *G. vaginalis* in vaginal flora (r=0,367;p<0,01 and r=0,324;p<0,01). Finding *A. vaginae* in vaginal flora closely correlated with the pathogenic findings of flora (r = 0,7, p <0,01) .

Discussion

Vaginal flora is delicate and dynamic system, with dominating inhabitant *Lactobacillus* species in the majority of women.[10,11,17,18]

This study included only small female population – first trimester pregnant women. In this study was confirmed by species-specific 16S rDNA gene PCR that *L. crispatus*, *L. inners* and *L. jensenii* are the most common species in Latvian pregnant women normal flora, that does not differ from the Finnish and Japanese data.[6,14,19] The incidence of *L. gasseri* in other similar studies match our data.[19,20]

L. jensenii detection in the normal flora group was higher while *L. inners* frequency did not differ between both groups, which was also confirmed in other similar studies.[14]

A. vaginae, G. vaginalis, Megasphera mainly prevailed in abnormal vaginal flora that did not differ from the global data. Interesting was the fact that one pathogenic bacteria correlated with each other, but there was no similar data in literature. [14, 21, 22] *A. vaginae* finding significantly correlated with pathogenic flora that is important because the literature describes a microorganism high resistance to metronidazole and susceptibility to clarithromycin. It means that in bacterial vaginosis treatment the use of metronidazole alone, which is effective against G. vaginalis, may not give the expected result.[4,14]

Comparing Grams staining method with pH measurements we found moderate correlation, but the vaginal pH measurements did not show all abnormal flora cases. However the pH test is specific enough, but with a low sensitivity, because not in all cases of abnormal flora pH is increased (≥ 4.5), so if only pH diagnostic method is used there may be many undiagnosed abnormal vaginal microflora cases, this makes it necessary to supplement this method of investigation with vaginal discharge microscopy.[1,3,17,18] Microscopy data were also compared with PCR. Although, the PCR can accurately identify the composition of bacteria in the vagina, but it is expensive and it has some drawbacks. There is no precise criteria for interpreting the normal or pathogenic, and using conventional polymerase chain method it can only prove the presence of microorganism in the vagina, but not the number of colony forming units, while Gram staining microscopy is simple vaginal flora diagnostic techniques that have established criteria for diagnostic the pathology.[1,3,17,18]

Unfortunately, in our country, such studies using PCR have not been performed so far. Our study group was relatively small, and analyzed cases only in Riga, therefore there is no clear vision on our region vaginal microflora nuances. During the study, we confronted with several problems and one of them was number of participants, although patient involvement lasted 6 months, the normal pH group included 45 of the expected 50 and a high pH group, only 20 of the 50 samples, this can be explained by the small population of our country. Patient involvement will continue

to reach the necessary number of respondents. The other problem of the study was the impossibility of using real-time PCR, because of study costs.

Overall this study gave a deeper insight into the occurring lactobacilli species in the Latvia. This is the root of future studies that will allow us to gain a deeper understanding of the vaginal flora in the Baltic region.

Conclusion

The most often isolated lactic acid bacteria in the vagina of pregnant Latvian women are *L. crispatus*, *L. jensenii* and *L. inners* less common are *L. gasseri* and *L. plantaris*. *L. jensenii* is the most common isolated lactic acid producing bacteria in normal flora of pregnant Latvian women. *A. vaginae, G. vaginalis, Megasphera* are the most common microbes found in pathogenic flora of vagina. Grams' staining method and vaginal pH measurements correlate with each other, but the vaginal pH measurements does not show all abnormal flora cases. In order to evaluate the vaginal environment for pregnant women, in addition to vaginal pH measurement is necessary for additional vaginal microflora diagnostics. Although the PCR can accurately identify the composition of bacteria in the vagina it is expensive, while the Grams staining microscopy is a simple vaginal flora change diagnostic methods. In order to better conclude on normal and abnormal vaginal microflora composition in Latvian women the further research are necessary.

Conflict of Interest: None declared.

References

1. Donders GGG. Definition and classification of abnormal vaginal flora. *Best practice & Research Clinical Obstetrics and Gynecology*, 2007; 1:1-18.
2. Rezeberga D. Dzemdniecība – Rīga: Medicīnas apgāds, 2009; 281.-282.
3. Donders GGG, Van Calsteren C, Bellen et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. BJOG.2009; 176:1315-1324.
4. Ferris MJ, Masztal A, Aldridge KE, Fortenberry JD, Fidel PL, Martin DH. Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. BMC Infect Dis. 2004; 4:5.
5. Vogel I, Thoresen P, Jeune B, et al. Acquisition and elimination of bacterial vaginosis during pregnancy: a Danish population-based study. Infect Dis Obstet Gyn. 2006; 2006: 1-6. doi 10.1155/IDOG/2006/94646.
6. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are

more conducive to the occurance of abnormal vaginal microflora. BMC Microbiol. 2009; 9:116.
7. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. Journal of Infectious Diseases. 1999; 180:1950-1956.
8. Anukam KC, Osazuwa EO, Ahonkhai I, Reid G. Lactobacillus vaginal microbiota of women attending a repoductive health care service in Benin city, Nigeria. Sex Transm Dis. 2006; 33:59-62.
9. Klatt TE, Cole DC, Eastwood DC, Barnabei VM. Fasctors associated with recurrent bacterial vaginosis. J Reprod Med. 2010; 55:55-61.
10. Kingsley CA, Emmanuel OO, Ahonkhai I, Reid G. 16S rRNA gene sequence and phylogenetic tree of *lactobacillus* species from the vagina of healthy Nigerian women. African Journal of Biotechnology. 2005; 4(11):1222-1227.
11. Bansal Garg K, Ganguli I, Das R, Talwar GP. Spectrum of *Lactobacillus* species present in healthy vagina of Indian women. Indian J Med Res. 2009; 129(6):652-657.
12. Vīberga I. Ginekoloģija: Rīga: Nacionālais apgāds. 2006; 148-151. lpp.
13. Rezeberga D, Lazdāne G, Kroiča J, Sokolova L, Teibe U. Women's reproductive tract infections: influence on duration and outcome of pregnancy Proc Latv Ac Scien. 2002; 56 (1/2):42 – 47.
14. Tamrakar R, Yamada T, Furuta I, et al. Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. *BMC Infectious Diseases.* 2007; 7:128.
15. Tynkkynen S, Satokai R, Saarela M, Mattila-Sandholm T, Saxelin M. Comparison of Ribotyping, Randomly Amplified Polymorphic DNA Analysis, and Pulsed-Field Gel Electrophoresis in Typing of *Lactobacillus rhamnosus* and *L. casei* Strains. Appl Environ Microbiol. 1999; 65(9):3908–3914.
16. Schwebke J, Lawing L. Prevalence of *Mobiluncus* spp Among Women With and Without Bacterial Vaginosis as Detected by Polymerase Chain Reaction. Sex Transm Dis. 2001; 28(4):195-199.
17. Donders GGG, Vereecken A, Salembier G. Van Bulck B, Spitz B. Assessment of lactobacillary flora in wet mount and fresh or delayed Gram's stain. Infect Dis Obstet Gynecol. 1996; 4:2-6.
18. Donders GGG, Vereecken A, Dekeersmaecker A. Van Bulck B, Spitz B. Wet mount reflects functional vaginal lactobacillary flora better than Gram stain. J Clin Pathol. 2000; 53:308-314.
19. Hernández-Rodríguez C, Romero-González R, Albani-Campanario M, Figueroa-Damián R, Meraz-Cruz N, Hernández-Guerrero C. Vaginal Microbiota of Healthy Pregnant Mexican Women is Constituted by Four Lactobacillus Species and Several Vaginosis-Associated Bacteria. Infect Dis Obstet Gynecol. 2011; 2011:1-9.
20. Redondo-Lopez V, Cook RL, Sobel JD. Emerging role of lactobacilli in the control and maintance of the vaginal bacterial microflora. Rev Infect Dis. 1990; 164(1):94-100.
21. Zhou X, Bent SJ, Schneider MG, Davis CC, Islam MR, Forney LJ. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiol. 2004; 150:2565-2573.

22. Zhou X, Brotman RM, Gajer P, Abdo Z, Schüette U, Ma S, Ravel J, Forney LJ. Recent advances in understanding the microbiolgy of the female reproductive tract and the causes of preterm birth. Infect Dis Ob Gyn. 2010; 2010:1-10. doi: 10.1155/2010/737425.

Table 1: PCR primer sequences and annealing temperatures

Microorganism	Primer sequence	PCR product	Annealing temperature
L. crispatus	LcrisF –AGC GAG CGG AACT AAC AGA TTTAC LcrisR –AGC TGA TCA TGC GATCTGCTT	154bp	65° [14]
L. jenseni	LjensF- AAGTCGAGCGAGCTTGCCTATAGA LjensR- CTTCTTTCATGCGAAAGTAGC	162bp	60° [14]
L. gasseri	LgassF – AGCGAGCTTGCCTAGATGAATTTG LgassR - TCTTTTAAACTCTAGACATGCGTC	170bp	63° [14]
L.inners	Liners-R –ACAGTTGATAGGCATCATCTG Liners-F - CTCTGCCTTGAAGATCGGAGTGC	155bp	65° [14]
L.plantaris	Lpla-3- ATTCATAGTCTAGTTGGAGGT Lpla-2- CCTGAACTGAGAGAATTTGA	248bp	64° [11]
L. rhamnosus	RhaI F - CTTGCATCTTGATTTAATTTTG RhaI R -CCGTCAATTCCTTTGAGTTT3	863bp	62° [15]
L. reuteri	Lreu-1- CAGACAATCTTTGATTGTTTAG Lreu-4 - GCTTGTTGGTTTGGGCTCTTC	303bp	64° [11]
G. vaginalis	GV1-F – TTACTGGTGTATCACTGTAAGG GV3-R - CCGTCACAGGCTGAACAGT	332bp	62° [14]
A. vaginae	AV-F - TAGGTCAGGAGTTAAATCTG AV-R - TCATGGCCCAGAAGACCGCC	156bp	62° [14]
Mobilluncus	Mob-AS- CGCAGAAACACAGGATTGCA Mob-S- GTGAACTCCTTTTTCTCGTGAA	450bp	60° [16]
Megasphera	MegaE-667R CCTCTCCGACACTCAAGTTCGA MegaE-456F GATGCCAACAGTATCCGTCCG	212bp	60° [14]
Leptotrichia/Sneathia	Lepto-395F CAATTCTGTGTGTGTGAAGAAG Lepto-646R ACAGTTTTGTAGGCAAGCCTAT	252bp	60° [14]

Table 2: The prevalence of microorganisms, detected with PCR, in vaginal flora, depending on the diagnostic method

	Total	*Nugent* score			pH level	
		0-3	*4-6*	*7-10*	< 4,5	≥4,5
Participant count	65	41	11	13	45	20
L. crispatus	47 (72%)	28 (68%)	9 (81%)	10 (76%)	33 (73%)	14 (70%)
L. jensenii	36 (55%)	26 (63%)	5 (45%)	5 (39%)	26 (58%)	10 (50%)
L. gasseri	22 (34%)	14 (34%)	5 (45%)	3 (23%)	18 (40%)	4 (20%)
L. inners	43 (66%)	27 (66%)	8 (73%)	8 (61%)	30 (67%)	13(65%)
L. reuteri	1 (2%)	1 (2%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)
L. plantaris	11 (17%)	7 (17%)	3 (27%)	1 (8%)	11 (24%)	0 (0%)
L. rhamnosus	1 (2%)	1 (2%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)
G. vaginalis	49 (75%)	30 (73%)	8 (73%)	11 (85%)	31 (69%)	18 (90%)
A. vaginae	12 (18%)	2 (5%)	2 (18%)	8 (61%)	4 (9%)	8 (40%)
Megasphera spp.	42 (65%)	25 (61%)	6 (55%)	11 (85%)	26 (58%)	16 (80%)
Leptotrichia spp.	41 (63%)	25 (61%)	5 (45%)	11 (85%)	28 (62%)	13 (65%)
Mobiluncus spp.	4 (6%)	2 (5%)	1 (9%)	1 (8%)	1 (2%)	3 (15%)

*** p<0,01**

Table 3: Nugent scoring system

Score	*Lactobacillus* morphotypes	*Gardnerella* and *Bacteroides* spp. morphotypes	*Mobiluncus*
0	4 + (> 30 per hpf)	0	0
1	3+ (5-30 per hpf)	1+ (< 1 per hpf)	1-2 +
2	2+ (1-5 per hpf)	2+ (1-5 per hpf)	3-4+
3	1+ (< 1 per hpf)	3+ (5-30 per hpf)	
4	0	4+ (> 30 per hpf)	

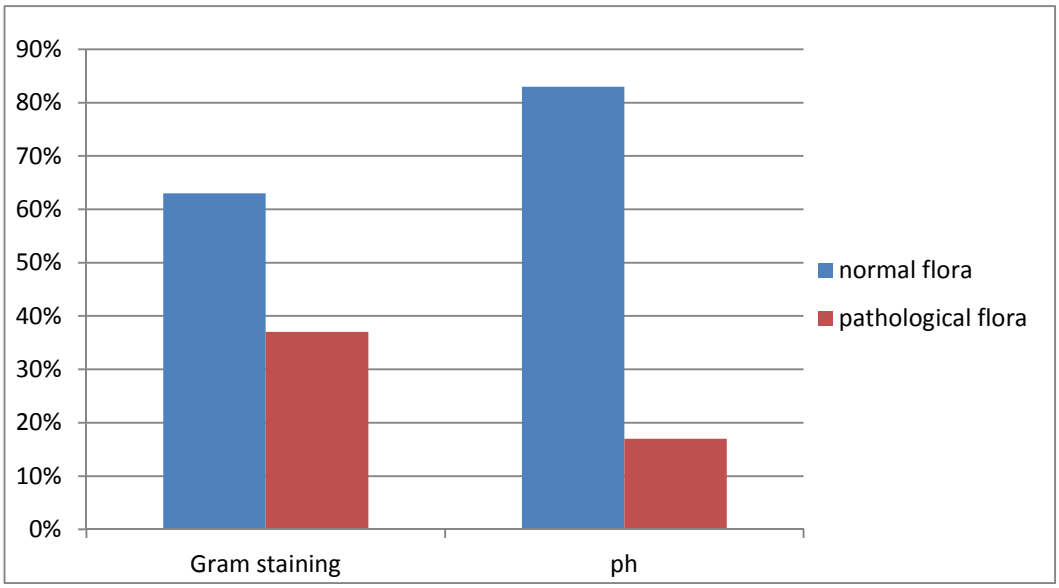

Diagram 1: The percentages of patients' distribution depending on diagnostic methods

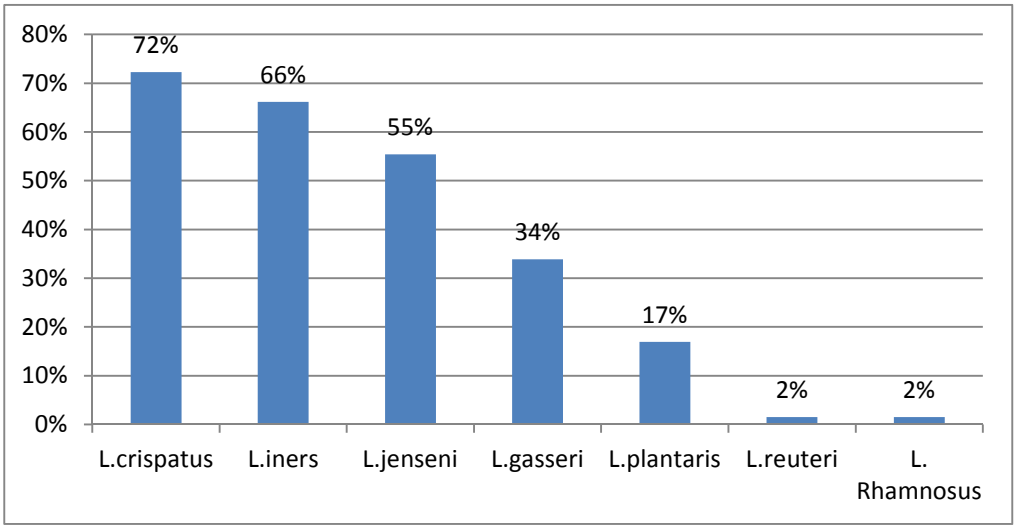

Diagram 2: Lactobacillus species in pregnant women