Eur. J. Clin. Microbiol. Infect. Dis., 1997, 16: 517–522

Relationship between Hydrogen Peroxide-Producing Strains of Lactobacilli and Vaginosis-Associated Bacterial Species in Pregnant Women

I.J. Rosenstein[1]*, E.A. Fontaine[1], D.J. Morgan[2], M. Sheehan[2], R.F. Lamont[2], D. Taylor-Robinson[1]

This study was conducted to determine the relationship between lactobacilli and bacterial species associated with bacterial vaginosis in pregnancy and the prevalence of H_2O_2-producing and non-producing strains of lactobacilli in pregnant women whose vaginal flora had already been analysed. Information was available for 174 pregnant women whose vaginal flora had been evaluated previously by examining gram-stained vaginal smears: 50 had grade III flora (bacterial vaginosis), 50 grade II flora, 41 flora graded as abnormal which then reverted to grade I (revertants) and 33 normal flora (controls). Lactobacilli were isolated from 19 of 50 women whose vaginal flora was grossly abnormal culturally and categorised as grade III by Gram staining. In 6 of these 50 women lactobacilli were isolated in large numbers, i.e. 10^5–10^6 cfu/ml. H_2O_2-producing strains of lactobacilli were isolated from 11 of 12 women with grade III flora who were randomly selected from this group. Thus, in those 11 women it appears that H_2O_2-producing lactobacilli had not protected them from developing bacterial vaginosis. Bacterial species associated with vaginosis were isolated in high numbers from a large proportion of women in the revertant and grade II groups in association with high counts of lactobacilli. Thus, in some women it is possible that a change to an abnormal flora could occur before the complete disappearance of lactobacilli. It is concluded that bacterial vaginosis may develop in some women despite the presence of H_2O_2-producing strains of lactobacilli and that other factors, as yet unidentified, might be conducive to the appearance of abnormal bacterial flora with progression to vaginosis.

The vaginal bacterial flora of healthy premenopausal women consists predominantly of *Lactobacillus* spp. (1). The role of lactobacilli in the control of the vaginal bacterial microflora has been discussed in several reviews (2, 3). These organisms are believed to play a protective role in the urogenital tract, guarding against infection by pathogens by stimulating the immune system, competing with other bacteria for adherence to epithelial cells and producing bactericidal compounds (4). Such compounds produced by lactobacilli include organic acids which lower the vaginal pH, low molecular weight bacteriocins and hydrogen peroxide H_2O_2 (2, 3).

In bacterial vaginosis, a condition characterized by an increased vaginal pH value and milky discharge, the healthy vaginal flora is replaced by a mixed flora of aerobic, anaerobic and microaerophilic species. An absence of H_2O_2-producing lactobacilli has been considered by one group as the reason for overgrowth of these organisms (5). They identified this as a risk factor for bacterial vaginosis on the basis of the isolation of H_2O_2-producing strains from the vagina of 96% of healthy women but from only 6% of women with vaginosis, whereas strains which did not produce H_2O_2 were isolated from 4% of healthy women and 36%

[1] MRC Sexually Transmitted Diseases Research Group, Imperial College School of Medicine at St Mary's, Norfolk Place, Paddington, London W2 1PG, UK.
[2] Department of Obstetrics and Gynaecology, Northwick Park Hospital, Harrow, Middlesex, UK.

Table 1: Isolation rates of vaginosis-associated bacterial species in the presence or absence of lactobacilli in women with abnormal vaginal flora.

	No. of revertants with indicated species		No. of grade II women with indicated species		No. of grade III women with indicated species	
Vaginosis associated species	With lactobacilli (n = 32)	Without lactobacilli (n = 9)	With lactobacilli (n = 33)	Without lactobacilli (n = 17)	With lactobacilli (n = 19)	Without lactobacilli (n = 31)
Corynebacterium spp.	18	3	22	7	15	26
Coagulase-negative staphylococci	25	6	25	16	11	26
Staphylococcus aureus	3	0	3	1	3	4
Gram-negative rods	2	1	2	3	1	5
Streptococcus spp.	5	1	10	9	9	19
Beta-haemolytic streptococci	1	0	1	5	4	2
Candida spp.	11	3	12	1	2	5
Anaerobic gram-positive cocci	6	2	8	12	13	26
Anaerobic gram-negative rods	6	4	10	10	13	22
Bifidobacterium spp.	14	5	17	12	18	29
Anaerobic gram-positive rods (not lactobacilli)	3	1	7	2	7	15
Mycoplasma hominis	2	5	3	4	10	18
Ureaplasma urealyticum	14	7	17	8	14	20

of women with vaginosis. In a more recent longitudinal study, women with H_2O_2-negative lactobacilli were twice as likely to acquire bacterial vaginosis as women with H_2O_2-producing strains (6).

We have recently described in detail the vaginal microbial flora of pregnant women with bacterial vaginosis and of healthy controls (7). This was part of a larger study to evaluate the effect of topically applied 2% clindamycin cream on the prevention of vaginosis-induced preterm delivery and early miscarriage in a double-blind placebo-controlled trial. In this study we determined the prevalence of H_2O_2-producing and H_2O_2-negative strains of lactobacilli in the various groups of women studied, and the relationship between lactobacilli and various bacterial species associated with vaginosis.

Materials and Methods

Study Population. The population of pregnant women studied has been described before (7). Briefly, samples for microbiological culture were taken from women attending the antenatal clinic at Northwick Park Hospital, where they had been diagnosed on the basis of a Gram stain of vaginal fluid as having an abnormal vaginal flora (grade II or III; 50 women in each category). The grading was performed as described by Hay et al. (8) who used the method of Spiegel (9) with the addition of an intermediate (grade II) category. Samples were also taken from 41 women who were described as revertants (7). These women had an abnormal flora on their first visit, but on returning to the clinic the abnormality was found to have resolved spontaneously and the Gram stain scored as grade I. Finally, samples were taken from 33 women who had normal grade I flora on the initial and subsequent screening and served as controls. The high vaginal and endocervical samples were subjected to a full microbiological examination as described previously (7).

Isolation of Lactobacillus Species. High vaginal swabs taken from the women described above were placed in bacteriological transport medium (7). The swab was agitated and squeezed thoroughly to dislodge its contents into the medium. The swab was then discarded and the medium stored at −70°C within one hour of inoculation and cultured within six months. For isolation of *Lactobacillus* spp., 0.1 ml of transport medium was plated onto two plates of Rogosa agar (Oxoid, UK) which were incubated microaerophilically (6% CO_2) for 48h and anaerobically (80% N_2, 10% CO_2, 10% H_2) for five days. Different colony types were recorded and subjected to semi-quantitation as described previously (7), the bacterial growth being recorded as ±, +, ++, +++ or ++++. A colony of each type was subcultured onto Rogosa agar and incubated anaerobically for three days. The growth was checked for purity and a Gram stain preparation made. Each strain of lactobacillus was stored in lysed defibrinated horse blood at −20°C until required for further testing.

Quantitation of Lactobacillus Species. High vaginal swab samples which were known to contain *Lactobacillus* spp. were recultured on Rogosa agar semi-quantitatively as described previously (7), and also quantitatively whereby appropriate dilutions of bacteriological transport medium were made in phosphate-buffered saline and 0.1 ml of each dilution spread onto Rogosa agar. Plates were incubated anaerobically for five days and the number of colonies counted and expressed as colony-forming units (cfu) per millilitre. Four samples in each growth category were subjected to quantitative culture, and the mean count (cfu/ml) was then used instead of the previous semi-quantitative value: ± was equivalent to $\leq 10^2$ cfu/ml, + to 10^3 cfu/ml, ++ to 10^4 cfu/ml, +++ to 10^5 cfu/ml, and ++++ to $\geq 10^6$ cfu/ml.

Tests for Hydrogen Peroxide Production. The ability of strains of lactobacilli to produce H_2O_2 was tested by the plate method described by McGroarty et al. (10), which is an adaptation of the method of Eschenbach et al. (5), and also by the quantitative tube method of Fontaine and Taylor-Robinson (11). For the plate method, stored strains were subcultured on Rogosa agar and a colony subcultured on deMann Rogosa

Table 2: Numbers of women with H_2O_2 producing lactobacilli among women randomly selected from each group.

Grade of vaginal flora	No. of women (n = 42)	No. of women with H_2O_2 positive or negative lactobacillus strains		
		H_2O_2 positive only (n = 23)	H_2O_2 positive and negative (n = 10)	H_2O_2 negative only (n = 9)
I (Controls)	10	7	1	2
I (Revertants)	10	5	3	2
II	10	4	2	4
III	12	7	4	1

Sharpe agar (Oxoid) containing 0.25 mg/ml of tetramethylbenzidine (Sigma, USA) and 0.1 mg/ml of horseradish peroxidase (Sigma). The plates were incubated under anaerobic conditions at 37°C for five days. They were then exposed to ambient air and colonies producing a blue pigment within 20 min of exposure were considered to be producing H_2O_2. To assess the strength of H_2O_2 production, the colour change was observed over time. A colour change occurring within the first 5 min of exposure to air was rated as a strong reaction, within 5 to 10 min as an intermediate reaction and within > 10 to < 20 min as a weak reaction. Any colour change occurring after 20 min was regarded as non-specific. For the tube method, stored strains were subcultured from lysed horse blood onto Rogosa agar and checked for purity. Colonies were scraped from the agar and inoculated into 1.5 ml of sterile phosphate-buffered saline to produce a heavy suspension (McFarland no. 3). Four drops (0.1 ml) of this suspension were used to inoculate 5 ml of peptonized milk yeast-dextrose-glycogen medium which was incubated at 37°C anaerobically with continuous agitation for three days (11). The supernatant fluid from the centrifuged broth culture was used to measure H_2O_2 iodometrically as described previously (11). A strong reaction was defined as producing ≥ 10 μg/ml H_2O_2, an intermediate reaction as producing > 3–< 10 μg/ml and a weak reaction as producing 1–3 μg/ml.

Results

Counts of Lactobacilli in Each Flora Group. Lactobacilli were isolated from 19 of 50 (38%) women with grade III flora, 33 of 50 (66%) women with grade II flora, 32 of 41 (78%) revertants and 30 of 33 (91%) controls. Of the women from whom lactobacilli were isolated, a substantial proportion in each group had high lactobacilli counts of 10^5–10^6 cfu/ml as follows: 6 of 19 (38%) women in the grade III group, 24 of 33 (73%) women in the grade II group, 27 of 32 (84%) revertants and 19 of 30 (63%) controls.

Isolation Rates of Vaginosis-Associated Bacterial Species in Women with Abnormal Flora. The bacterial flora of the women with vaginosis (grade III), women with grade II flora, revertants and controls has been described in detail elsewhere (7). For the three groups of women with abnormal flora, the data were analysed to determine if any differences existed between the vaginal flora of women in whom lactobacilli were present and those in whom lactobacilli were absent. The results are shown in Table 1.

In the revertant group, women from whom lactobacilli were not isolated were more likely to have anaerobic gram-negative rods, *Mycoplasma hominis* and *Ureaplasma urealyticum* than were women from whom lactobacilli were isolated. Other organisms associated with vaginosis were isolated with equal frequency. In the grade II group, beta-haemolytic streptococci, anaerobic gram-positive cocci, anaerobic gram-negative rods, *Bifidobacterium* spp. and *Mycoplasma hominis* were more likely to be isolated from women in whom lactobacilli were absent than from women who had lactobacilli. However, in the grade III group, all vaginosis-associated bacterial species other than anaerobic gram-positive cocci were isolated with equal frequency from women with or without lactobacilli. Thus, lactobacilli may be present even when the vaginal flora is grossly abnormal.

Table 3: Numbers of H_2O_2 positive and negative strains of lactobacilli isolated from women with various grades of vaginal flora and the strength of the H_2O_2 reaction.

Grade of vaginal flora	No. of women	No. of strains tested	No. (%) of H_2O_2 negative strains	No. (%) of H_2O_2 positive strains	No. of H_2O_2 positive strains by reaction		
					Weak reaction	Intermediate reaction	Strong reaction
I (Controls)	10	20	4 (20)	16 (80)	1	3	12
I (Revertants)	10	20	7 (35)	13 (65)	2	2	9
II	10	17	7 (41)	10 (59)	1	4	5
III	12	19	7 (37)	12 (63)	–	1	11

Counts of Lactobacilli Associated with Abnormal Flora. The number of lactobacilli isolated was assessed in relation to the presence or absence of bacterial species associated with vaginosis in each group of women with abnormal flora. In the revertant group, lactobacilli were isolated in large numbers (10^5–10^6 cfu/ml) from a large proportion of women from whom vaginosis-associated species were also isolated, namely 20 of 25 women with coagulase-negative staphylococci, 13 of 14 women with *Bifidobacterium* spp., all of 6 women with anaerobic gram-positive cocci and 5 of 6 women with anaerobic gram-negative rods. Furthermore, in the majority of cases even where lactobacilli were isolated in large numbers (10^5–10^6 cfu/ml), the vaginosis-associated species were also isolated in large numbers from 10 of the 13 women with *Bifidobacterium* spp., 4 of the 6 women with anaerobic gram-positive cocci, but only 1 of 5 women with anaerobic gram-negative rods. In women in the grade II group with 10^5–10^6 cfu/ml lactobacilli, coagulase-negative staphylococci were isolated from 19 of 25 women, *Bifidobacterium* spp. from 11 of 17 women, anaerobic gram-positive cocci from 6 of 8 women, anaerobic gram-negative rods from 7 of 10 women and *Mycoplasma hominis* from all of 3 women. In these women, 4 of the 11 with *Bifidobacterium* spp., 3 of the 6 with anaerobic gram-positive cocci, 3 of the 7 with anaerobic gram-negative rods and 1 of the 3 with *Mycoplasma hominis* had high counts of the organisms. In the grade III group, the number of women from whom lactobacilli were isolated in large numbers in association with abnormal bacterial species was lower than in the other two groups. However, in a substantial proportion of women, lactobacilli were isolated in large numbers (10^5–10^6 cfu/ml) in association with vaginosis-associated species, namely 4 of 11 women with coagulase-negative staphylococci, 6 of 18 women with *Bifidobacterium* spp., 5 of 13 women with anaerobic gram-positive cocci, 4 of 13 women with anaerobic gram-negative rods and 5 of 10 women with *Mycoplasma hominis*. In these women, 3 of the 6 with *Bifidobacterium* spp., 1 of the 5 with anaerobic gram-positive cocci, 2 of the 4 with anaerobic gram-negative rods and 3 of the 5 with *Mycoplasma hominis* had high counts of the organisms.

H_2O_2 Production by Strains of Lactobacilli in Women with Abnormal Flora. A total of 76 strains of lactobacilli which had been stored were tested for H_2O_2 production by both the plate and tube methods. These strains were isolated from 10 controls (20 strains), 10 revertants (20 strains), 10 grade II women (17 strains) and 12 grade III women (19 strains). The results obtained by the two methods were in agreement for 65 of 76 strains tested, the remaining 11 strains being positive in the plate test but negative in the tube test, which may be due to differences in the culture conditions of the tests (11). However, there was a trend for the speed of reactivity in the plate test to be proportional to the amount of H_2O_2 produced, in that of 37 strains producing a strong reaction in the plate test, 17 produced a strong reaction and 6 an intermediate reaction in the tube test. However, for further analysis, a strain was considered to be an H_2O_2-producer if it was positive in the plate test even though negative in the tube test. As shown in Table 2, H_2O_2-producing strains were isolated from 8 of 10 women in the control group, 8 of 10 women in the revertant group, 6 of 10 women in the grade II group and, surprisingly, from 11 of 12 women in the grade III group. However, when the total number of strains isolated from each group of women was assessed for H_2O_2 production (Table 3), the percentage of H_2O_2-producing strains was higher in the controls (80%) than the revertants (65%), but the percentage of positive strains in the grade II (59%) and grade III (63%) groups was similar to that in the revertants. The ratio of positive to negative strains was similar in the revertant (1.9:1), grade II (1.4:1) and grade III (1.8:1) groups but higher in the control group (4:1).

Numbers of H_2O_2-Producing Lactobacilli in Women with Abnormal Flora and the Strength of H_2O_2 Production. The distribution of strains of

Table 4: Strength of H_2O_2 production related to the lactobacilli count.

Grade of vaginal flora	No. of strongly H_2O_2 producing strains isolated related to lactobacilli count (cfu/ml)*				
	$\geq 10^2$	10^3	10^4	10^5	10^6
I (Controls)	–	2(2)	1 (1)	1 (1)	8 (16)
I (Revertants)	–	–	0 (2)	5 (5)	5 (13)
II	–	0 (2)	0 (1)	0 (1)	5 (13)
III	3 (4)	2 (2)	2 (3)	2 (7)	1 (3)

* Total number of strains tested in brackets.

lactobacilli in each group of women according to the strength of H_2O_2 production is shown in Table 3. In the grade III group, where the lactobacilli were often H_2O_2-positive (12 of 19 strains tested), nearly all were strong producers of H_2O_2 (11/12; 92%). The proportion of strains which were strong producers in the other three groups was 75% (12/16) in controls, 69% (9/13) in revertants and 50% (5/10) in grade II women.

When the relative amount of H_2O_2 produced was considered in relation to the lactobacilli counts (Table 4), in women with grade III flora from whom lactobacilli were isolated in low numbers (10^2–10^4 cfu/ml), 7 of 9 strains (from 6 women) were strong producers of H_2O_2. Where lactobacilli were isolated in large numbers (10^5–10^6 cfu/ml), 3 of 10 strains (from 6 women) were strong producers. Thus, lactobacilli may be strong producers of H_2O_2, even when occurring in low numbers, as they often do in women with grade III flora.

Discussion

Bacterial vaginosis is commonly defined as a syndrome characterised by an absence of lactobacilli in the vaginal microbial flora and their replacement by a mixture of aerobic, anaerobic and microaerophilic species, the organisms of which exist in large numbers. The complete disappearance of lactobacilli may represent the very end of a transition from normal flora, consisting predominantly of lactobacilli, to a grossly abnormal flora as represented by some women who have grade III flora in which there are no lactobacilli at all. The failure to recover lactobacilli from a small proportion of the revertant group could be due to a return to clinical normality prior to regaining a normal flora (7).

It is necessary to be cautious when drawing conclusions from cross-sectional data rather than from a longitudinal cohort study. However, our data show that in some women in the revertant and grade II groups, vaginosis-associated species were isolated together with large numbers of lactobacilli, so that it seems possible, in some cases at least, that abnormal bacterial species start to appear and increase in numbers prior to the disappearance of the lactobacilli. Furthermore, we agree with Reid et al. (2) that lactobacilli can be isolated from a substantial proportion of women with grade III flora, and that in cases where the lactobacilli persist, they often do so in large numbers.

In women who had grade III flora, the isolation of bacteria belonging to the abnormal flora was not affected by the presence or absence of lactobacilli. This raises the question as to what protects against vaginal overgrowth of abnormal bacterial species. It is commonly believed that the presence of H_2O_2-producing lactobacilli may provide such protection (5). We did not test for H_2O_2 production in all the strains isolated in this study. However, we found that when lactobacilli randomly selected from women with grade III flora were tested, they were as likely to be producing H_2O_2 as not, and that these strains were also likely to be strong producers of H_2O_2. Thus it seems that these women were not protected by the presence of H_2O_2-producing vaginal lactobacilli. Similar isolation rates of H_2O_2-positive and negative lactobacilli have been found in non-pregnant women with bacterial vaginosis (12). In our study, the proportion of H_2O_2-producing strains was reduced. However, the ratio between positive and negative strains was higher in the controls (4:1) than in the revertants (1.9:1) but was at the same level in the other two groups.

The data presented here and the observations made in our previous study (7), indicate that the vaginal flora is not static but can convert from a normal state to a grossly abnormal state and back again. Lactobacilli tend to disappear in women whose vaginal flora is recorded as grade III, but those lactobacilli which remain may do so in large numbers, are not necessarily H_2O_2-negative, and may produce H_2O_2 in large amounts. In some women, the flora reverts spontaneously to normal, possibly before the lactobacilli completely disappear, and there may be certain factors relating to the individual women's susceptibility to bacterial vaginosis which cause the flora to revert to normal or progress to a grossly abnormal state. This may affect the women's response to antibiotic therapy for vaginosis. It is currently not known what factors bring about the appearance of the abnormal bacterial species and progression to and regression of vaginosis. It is clear, however, that emphasis should be placed on determining the nature, physiological or otherwise, of these factors.

References

1. Hill GB, Eschenbach DA, Holmes KK: Bacteriology of the vagina. Scandinavian Journal of Urology and Nephrology 1985, Supplement 86: 23–39.
2. Reid G, Bruce AW, McGroarty JA, Cheng KJ, Costerton JW: Is there a role for lactobacilli in prevention of uro-

genital and intestinal infections? Clinical Microbiology Reviews 1990, 3: 335–344.
3. Redondo-Lopez V, Cook RL, Sobel JD: Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Reviews of Infectious Diseases 1990, 12: 856–872.
4. McGroarty JA: Probiotic use of lactobacilli in the human female urogenital tract. FEMS Immunology and Medical Microbiology 1993, 6: 251–264.
5. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmes KK: Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. Journal of Clinical Microbiology 1989, 27: 251–256.
6. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wølner-Hanssen P, Holmes KK: Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. Journal of Infectious Diseases 1996, 174: 1058–1063.
7. Rosenstein IJ, Morgan DJ, Sheehan M, Lamont RF, Taylor-Robinson D: Bacterial vaginosis in pregnancy – distribution of bacterial species in different Gram stain categories of the vaginal flora. Journal of Medical Microbiology 1996, 45: 120–126.
8. Hay PE, Lamont RF, Taylor-Robinson D, Morgan DJ, Ison C, Pearson J: Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. British Medical Journal 1994, 308: 295–298.
9. Spiegel CA, Amsel R, Holmes KK: Diagnosis of bacterial vaginosis by direct Gram stain of vaginal fluid. Journal of Clinical Microbiology 1983, 18: 170–177.
10. McGroarty JA, Tomeczek L, Pond DG, Reid G, Bruce AW: Hydrogen peroxide production by *Lactobacillus* species. Correlation with susceptibility to the spermicidal compound Nonoxynol-9. Journal of Infectious Diseases 1992, 165: 1142–1144.
11. Fontaine EAR, Taylor-Robinson D: Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacillus. Journal of Applied Bacteriology 1990, 69: 326–331.
12. Fontaine EA, Claydon E, Taylor-Robinson D: Lactobacilli from women with or without bacterial vaginosis and observations on the significance of hydrogen peroxide. Microbial Ecology in Health and Disease 1996, 9: 135–141.