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# Influence of the Spermicidal Compound Nonoxynol-9 on the Growth and Adhesion of Urogenital Bacteria in vitro

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**Abstract.** Lactobacilli and uropathogenic bacteria isolated from the female urogenital tract were tested for their susceptibility to nonoxynol-9. Nonoxynol-9 is a spermicidal compound, generally used at a concentration of 5% in cream and 12.5% in foam. The growth of 67% of fresh, vaginal lactobacillus isolates was inhibited by concentrations of nonoxynol-9 between 0.1% and 1.0%; these were termed sensitive. Of a total of 47 lactobacilli from various sources, 55% were found to be sensitive to nonoxynol-9, being bacteriostatic for 42% of these isolates and bactericidal for the remaining 58% at N-9 concentrations $\geq$ 1.0%. The remaining lactobacilli and 96% (48/50) of uropathogenic organisms had minimal inhibitory concentrations of $\geq$25% for nonoxynol-9. Inhibition of the lactobacilli did not appear to be species specific nor related to the source of the lactobacilli. The adhesion of Gram-positive bacteria, namely lactobacilli and enterococci, to HeLa cells in tissue culture was significantly increased over 60 min in the presence of physiologically used concentrations of nonoxynol-9; however, adhesion of *Escherichia coli* was not affected. We believe that nonoxynol-9 has the potential to increase susceptibility to urinary tract infection in women using spermicidal preparations for contraception by inhibiting the growth of lactobacilli, which are believed to have a protective function in the vagina, and allowing overgrowth of uropathogenic bacteria.

The role of the normal microbial flora of the vagina in the maintenance of a healthy state is presently not completely understood. However, *Lactobacillus* species are believed to play a part in protection against colonization by pathogenic microorganisms. Women with a history of urinary tract infection have a urogenital flora dominated by pathogens [12], whereas lactobacilli predominate in healthy women [4, 17]. The ecological balance of the vagina can be upset in a number of ways, including the prolonged use of antibiotics [11] and pregnancy.

Diaphragm use has been implicated as a predisposing factor in recurrent urinary tract infection [6–8]. A number of studies have found that women using this form of contraception are between 1.5 [16] and 4.1 [5] times more likely to develop a urinary tract infection. It has been suggested that the increased pressure of the diaphragm on the female urethra may be in part responsible for this [7]. However, the diaphragm is generally used in conjunction with a spermicidal cream or foam, and these preparations have been found to have a bactericidal effect on gonococci [1], *Treponema pallidum* [14], *Chlamydia trachomatis* [3], and a weak effect on *Lactobacillus acidophilus* [15], as well as a virucidal effect against *Herpes simplex* I and II and HIV [10].

Nonoxynol-9 (N-9) is a membrane-active detergent and the active ingredient of many spermicidal creams and foams. It is incorporated in these preparations at 5% and 12.5% (wt/wt) respectively. It immobilizes sperm by disrupting the cell membrane and acts similarly on bacteria and viruses. This study has examined the effect, in vitro, of N-9 over a range of concentrations on the growth and adhesion of lactobacilli and uropathogenic bacteria to human epithelial cells.

## Materials and Methods

**Bacteria.** Vaginal swabs were obtained from 44 premenopausal women with and without vaginitis, who used a variety of birth control methods. Smears from the swabs were Gram stained, examined for the presence of lactobacilli, and plated onto lactobacilli MRS agar (MRS) and brain–heart infusion agar supplemented with 2% yeast extract (BYE agar). The plates were incubated at

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Table 1. Minimum inhibitory concentration of N-9 for lactobacilli

| *Lactobacillus* species | Number of strains | Minimum inhibitory concentration <1.0% | >25.0% |
|---|---|---|---|
| *Lactobacillus* spp.[a] | 18 | 12 (67%) | 6 (33%) |
| *L. acidophilus* | 12 | 8 (67%) | 4 (33%) |
| *L. plantarum* | 2 | 1 (50%) | 1 (50%) |
| *L. casei ss rhamnosus* | 8 | 1 (13%) | 7 (88%) |
| *L. casei ss alactosus* | 1 | 0 (0%) | 1 (100%) |
| *L. jensenii* | 3 | 3 (100%) | 0 (0%) |
| *L. fermentum* | 2 | 1 (50%) | 1 (50%) |
| *L. brevis* | 1 | 0 (0%) | 1 (100%) |

[a] Fresh vaginal isolates, not speciated.

Table 2. Lactobacillus sensitivity to N-9: relationship to source of organisms

| Source of lactobacilli | Number of isolates | Sensitive[a] | Resistant[b] |
|---|---|---|---|
| Vagina[c] | 18 | 12 (67%) | 6 (33%) |
| Urogenital tract[d] | 25 | 12 (48%) | 13 (52%) |
| Dairy | 1 | 0 (0%) | 1 (100%) |
| Chicken | 3 | 2 (67%) | 1 (33%) |
| Total | 47 | 26 (55%) | 21 (45%) |

[a] N-9 MIC < 1%.
[b] N-9 MIC ≥ 25%.
[c] Fresh clinical isolates.
[d] Stored laboratory strains.

Table 3. Minimum inhibitory concentration of N-9 for uropathogenic bacteria

| Microorganism | Number of strains tested | N-9 M.I.C. |
|---|---|---|
| *Escherichia coli* | 13 | >25% |
| *Pseudomonas aeruginosa* | 4 | 3.0%; 25% |
| *Providencia stuartii* | 1 | 25% |
| *Citrobacter freundii* | 1 | 25% |
| *Klebsiella pneumoniae* | 4 | 25% |
| *Proteus mirabilis* | 5 | >25% |
| *Enterococcus faecalis* | 20 | 25% |
| *Staphylococcus saprophyticus* | 1 | >25% |
| *Staphylococcus* spp. | 1 | >25% |
| *Corynebacterium* spp.[a] | 1 | 1.6 |

[a] Commensal organism.

37°C for 18 h in 5% $CO_2$. Organisms recovered from the plates were identified as lactobacilli with the API rapid CH kit and stored at −70°C in MRS broth plus 20% (wt/vol) glycerol. Freeze-dried lactobacillus isolates from the laboratory collection originally isolated from chicken, dairy, and human sources were also cultured on MRS agar and broth. Uropathogenic bacteria previously isolated from the urine of women with urinary tract infections were cultured on BYE medium.

**Determination of minimum inhibitory concentration (M.I.C.) of nonoxynol-9.** Doubling dilutions of N-9 (Ortho Pharm., Canada) were made in MRS (pH 6.5) and BYE broth (pH 7.4) ranging from 0.1% to 25% (wt/vol). The pH of the N-9-supplemented medium was adjusted to the same level as unsupplemented medium where necessary. Lactobacillus isolates were subcultured three times prior to testing in the N-9 broths, and uropathogenic bacteria were simply incubated overnight. The cultures were washed three times in phosphate-buffered saline, pH 7.1 (PBS), and resuspended to a concentration of $10^7$ cells $ml^{-1}$. Aliquots of 50 $\mu$l were added to test tubes in triplicate containing 3 ml of either medium alone (control) or medium plus N-9, mixed thoroughly, and incubated for 18 h at 37°C. The tubes were scored for growth, and the M.I.C. was recorded as the lowest concentration demonstrating no growth. Aliquots of 50 $\mu$l were transferred from N-9/MRS tubes showing no growth to 3 ml of fresh MRS, incubated for 18 h at 37°C, and examined for turbidity.

**Preparation of HeLa cells.** HeLa cells (ATCC CCL2) were cultured in monolayers on glass coverslips in multiwell tissue culture trays in Eagle's minimum essential medium containing glutamine (Gibco), supplemented with 2.2 g $L^{-1}$ $NaHCO_3$, 10% fetal bovine serum, 100 units penicillin $ml^{-1}$, and 100 mg streptomycin $ml^{-1}$. Confluent monolayers formed after 3–4 days of incubation at 37°C in an atmosphere of 10% $CO_2$.

**Adhesion assay.** The adhesion of strains of lactobacilli, enterococci, and *E. coli* to HeLa cells was performed according to the method of Samaranayake and MacFarlane [13]. Washed HeLa-cell monolayers were incubated with the bacteria, which were cultured as previously described, washed three times in PBS, and resuspended to a concentration of $10^8$ cells $ml^{-1}$ in Dulbecco's PBS, pH 7.1 (0.1 g $CaCl_2$ anhd., 0.2 g KCl, 0.2 g $KH_2PO_4$, 0.1 g $MgCl_2 \cdot 6H_2O$, 8.0 g NaCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$, in 1L distilled $H_2O$). N-9 was incorporated in the adhesion assays at a final concentration of 0, 5.0%, or 12.5% (wt/vol), and the tissue culture trays were incubated at 37°C in an orbital shaker at 60 rpm for 60 min. The monolayers were then washed in Dulbecco's PBS, fixed in 10% formal saline, and Gram stained. The number of bacteria adherent to 50 HeLa cells was counted by light microscopy, and each condition was assayed in triplicate. The viability of bacteria after 60 min of incubation in either PBS or N-9 was determined by plating serial dilutions of the bacteria onto appropriate media.

**Statistics.** The two-tailed, unpaired t-test was used to detect significant differences between control and N-9-influenced bacterial adhesion to HeLa cells. The chi-squared test was used to compare the proportions of N-9 sensitive and resistant strains of each *Lactobacillus* spp., to determine whether this property was species specific.

## Results

**Nonoxynol-9 minimum inhibitory concentrations.** N-9 M.I.C.s for a total of 47 lactobacilli from various

Table 4. Influence of 5 and 12.5% N-9 on adhesion of bacteria to HeLa cells in vitro

| Bacteria | Control | 5% N-9 | A.I.[a] | 12.5% N-9 | A.I. |
|---|---|---|---|---|---|
| *L. casei* GR-1 | 0.15 ± 0.06[b] | 1.27 ± 0.19 | 8.7*[c] | 0.71 ± 0.15 | 4.9* |
| *L. fermentum* B-54 | 0.09 ± 0.02 | N.D.[d] | | 0.27 ± 0.06 | 2.9* |
| *L. acidophilus* T-13 | 0.86 ± 0.07 | 0.55 ± 0.06 | 0.6* | 0.43 ± 0.06 | 0.5* |
| *Lactobacillus* spp. 1 | 0.03 ± 0.02 | 0.32 ± 0.08 | 11.9* | 0.21 ± 0.07 | 7.9* |
| *Lactobacillus* spp. 2 | 0.02 ± 0.01 | N.D. | | 0.42 ± 0.08 | 21.0* |
| *Lactobacillus* spp. 4 | 0.02 ± 0.02 | 0.22 ± 0.06 | 11.0* | 0.09 ± 0.04 | 4.5 |
| *Ent. faecalis* 1030 | 1.67 ± 0.33 | 6.79 ± 0.64 | 4.1* | 7.62 ± 0.88 | 4.6* |
| *Ent. faecalis* 1470 | 0.57 ± 0.14 | 1.81 ± 0.33 | 3.2* | 1.61 ± 0.17 | 2.8* |
| *E. coli* 12269 | 1.75 ± 0.36 | N.D. | | 1.25 ± 0.16 | 0.7 |
| *E. coli* 12313 | 1.35 ± 0.30 | 1.68 ± 0.38 | 1.2 | 1.18 ± 0.18 | 0.8 |

[a] Adhesion index, adhesion in the presence of N-9 relative to control (N-9 adhesion value/control adhesion).
[b] Mean no. adherent bacteria per HeLa cell ± S.E.M., n = 150.
[c] $P < 0.005$ versus control by student t-test.
[d] Not determined.

sources are shown in Table 1; 55% (26/47) of the strains were inhibited by 0.1% to 1.0% N-9, and 45% (21/47) were able to grow in the presence of 25% N-9, the maximum concentration tested. Of the 26 strains that showed suppression of growth in concentrations of N-9 > 1.0%, 15 were rendered nonviable in concentrations of N-9 ≥ 1.0%. The growth of the remaining 11 strains was simply suppressed in the presence of N-9. There was no correlation between particular species (Table 1) or the source of the bacteria and N-9 susceptibility of the lactobacilli (Table 2). The majority of a range of uropathogenic bacteria had MICs of ≥25% N-9 (Table 3).

**Adhesion of lactobacilli and uropathogenic bacteria in the presence of nonoxynol-9.** In general, N-9 was found to significantly increase the adhesion of Gram-positive bacteria to HeLa cells over 60 min of incubation, with the exception of *L. acidophilus* T-13, which had a baseline adhesion much higher than any of the other lactobacilli tested. The increased adhesion was not dose related. Adhesion of *Escherichia coli* was not significantly affected, although there was a trend towards slightly decreased adhesion. The results obtained from physiologically used concentrations, 5 and 12.5%, are shown in Table 4. Bacterial viability was not significantly affected in the presence of N-9 over 60 min of incubation.

## Discussion

This study has concentrated on the effect of N-9 on lactobacilli and potential uropathogenic bacteria. Fresh vaginal isolates of *Lactobacillus* spp. could be split into two groups according to their N-9 M.I.C.s; 67% (12/18) had M.I.C.s for N-9 between 0.1 and 1.0%; the remainder grew in 25%, twice the maximum concentration used for contraceptive purposes. Organisms with an M.I.C. between 0.1% and 1.0% were termed sensitive, those with M.I.C.s ≥25% were termed resistant. Of the sensitive lactobacilli, N-9 exerted a bactericidal effect on 58% of the organisms and bacteriostasis with the remaining lactobacilli.

The M.I.C.s for both lactobacilli and uropathogenic microorganisms were determined in media that ensured optimal growth of the organism, rather than attempting to mimic conditions within the vagina. Gram-negative uropathogens are unable to grow under the conditions of low pH found in the normal, healthy vagina. During episodes of urinary tract infection, colonization of the vagina with these organisms increases, along with a rise in vaginal pH. The pH of the vagina has been found to rise from the normal levels of around pH 4.0, to pH values > 5.5 [8]. It seems likely that there will be some variation in pH levels within the vagina during the onset of infection, where the pathogenic organisms create microniches of higher pH.

Lactobacilli from chicken and clinical sources included both N-9-sensitive and -resistant organisms. There was no apparent correlation between the source of the organism and susceptibility to N-9. The ability to grow in the presence of high concentrations of N-9 is not unique to lactobacilli from the urogenital tract, and does not require prior exposure to N-9.

The continued presence of N-9 in the vagina would be expected to exert a selective pressure on the microbial flora, suppressing or killing sensitive

lactobacilli and resulting in a vaginal flora dominated by either pathogenic organisms or N-9-resistant lactobacilli. Studies have shown that total number of lactobacilli decreases, and there is increased colonization of the urogenital tract by coliform bacteria in women using diaphragms plus spermicide [6, 8]. This suggests that the normal urogenital flora may be dominated by N-9-sensitive lactobacilli. Gram-positive and -negative uropathogens were not inhibited by the presence of high concentrations of N-9 in growth medium, with the exception of two out of four strains of *Pseudomonas aeruginosa*. A single isolate of *Corynebacterium* species, which is a commensal of the urogenital tract, was found to be sensitive to N-9, further evidence of the displacement of normal flora organisms.

The ability of Enterobacteriaceae to grow in high concentrations of detergent has been previously reported [9]. Kramer and Nickerson showed that *Enterobacter cloacae* could grow in the presence of 10% sodium dodecyl sulfate.

There is evidence that toxic shock syndrome is related to the prolonged retention of a diaphragm used in conjunction with spermicide [2]. The *Staphylococcus* species investigated in this study were resistant to N-9, confirming that spermicide could potentially contribute to initiation of this disease by fostering overgrowth of this organism.

The adhesion of lactobacilli to HeLa cells in tissue culture was found to be of a very low level in comparison with uropathogenic bacteria. Adhesion was influenced by the presence of N-9 in the assay system. The relative adhesion of the Gram-positive bacteria tested (lactobacilli and enterococci), with one exception, was significantly increased by between 2.8 and 21 times. The increased adhesion was not dose related. Adhesion of the Gram-negative organisms, namely *E. coli*, was not affected by the presence of up to 12.5% N-9. If the increases observed were simply owing to a general increase in hydrophobicity of the HeLa cells or the bacteria, the adhesion of all organisms including *E. coli* would be expected to increase. It may be that the Gram-positive cell wall accumulates N-9 more efficiently than the Gram-negative envelope, accounting for the difference in adhesion. The assay was limited to a 60-min incubation and a neutral pH, to prevent loss of viability of HeLa cells, and may not accurately reflect the situation in vivo, where the vaginal pH is around 4.5 and the epithelium is in contact with spermicide for 6 h and longer. In addition many of the lactobacilli tested in the adhesion assay were shown to be extremely sensitive to N-9 and would not be expected to survive in the vagina. However, the increases in adhesion of N-9-resistant uropathogenic organisms suggest a potential mechanism for increasing the incidence of urinary tract infection in women using spermicide for contraception.

In conclusion, the vaginal lactobacillus population contains both sensitive and resistant individuals. N-9 has the potential to alter the microbial flora of the vagina by killing or suppressing the growth of the protective lactobacilli and allowing overgrowth, as well as possibly increased adhesion of potential uropathogens.

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