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A study of the susceptibility of three species of primate to vaginal colonization with *Gardnerella vaginalis*

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Summary. In an attempt to develop an animal model of *Gardnerella*-associated vaginitis, several strains of *Gardnerella vaginalis* were inoculated into the lower genital tract of female pig-tailed macaques, tamarins and chimpanzees. *G. vaginalis* was not recovered from either tamarins or chimpanzees, but was recovered from each of 10 pig-tailed macaques inoculated with either of two freshly isolated *Gardnerella* strains, colonization persisting for 11–39 days. Examination of Gram-stained vaginal smears obtained from infected pig-tailed macaques failed to demonstrate clue cells, a feature which is pathognomonic of *Gardnerella*-associated vaginitis in humans. Other features characteristic of non-specific vaginitis, namely an increase in vaginal pH, and an increase in the ratio of succinate to lactate (S/L ratio) in vaginal fluid were not found. However, the physiology of the macaque vagina was found to be different from that of the human, the vaginal pH and S/L ratio of uninfected macaques both being higher than that seen in humans. The physiological differences between the macaque and human vagina may be due, in part, to a difference in their anaerobic vaginal flora. While these inter-species differences in vaginal physiology and microbiology limit the relevance of the pig-tailed macaque as a model of *Gardnerella*-associated vaginitis, the ease with which macaques are colonized with *G. vaginalis* may prove useful in studying bacterial adhesion and local immunity.

Keywords: non-specific vaginitis, *Garderella vaginalis*, clue cells

In 1955, Gardner & Dukes reported the isolation of a hitherto unclassified bacterium from women suffering from so-called 'non-specific' vaginitis (NSV), a condition characterized by foul-smelling vaginal discharge, an elevated vaginal pH and the presence of epithelial cells covered with coccobacilli (clue cells). The micro-organism which they described has since been given the name *Gardnerella vaginalis* (Greenwood & Pickett 1980). Although Gardner & Dukes suggested that *G. vaginalis* was the causative agent of NSV, the role that the micro-organism plays in the pathogenesis of the disease is still a matter of some controversy. Gardner & Dukes (1955) reported that *G. vaginalis* was readily isolated from women with NSV, but was not isolated from women

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with normal vaginal secretions. While this association between the isolation of *G. vaginalis* and NSV has been supported by some workers (Pheifer *et al.* 1978; Spiegel *et al.* 1980; Taylor *et al.* 1982), the micro-organism has been found in the normal vaginal flora to such an extent by some workers (Levison *et al.* 1977, 1979; McCormack *et al.* 1977; Pheifer *et al.* 1978; Spiegel *et al.* 1980; Amsel *et al.* 1983; Ison *et al.* 1983) for them to question its role in NSV. Further evidence supporting the concept that *G. vaginalis* produces NSV was provided by the observation that the disease developed in a proportion of volunteers inoculated with pure cultures of the micro-organism (Gardner & Dukes 1955; Criswell *et al.* 1969). Recently, however, it has been reported that there is also an increase in the anaerobic vaginal flora of women with NSV (Pheifer *et al.* 1978; Spiegel *et al.* 1980; Taylor *et al.* 1982), and Chen *et al.* (1979) showed that various amines which are present in the vaginal fluid of NSV patients, and which may be responsible for the malodour, could be reproduced *in vitro* by *Bacteroides* species, but not by *G. vaginalis*. Chen *et al.* (1979) also suggested that a symbiotic relationship existed between *G. vaginalis* and *Bacteroides* species since *G. vaginalis* produced and released high concentrations of pyruvic and amino acids which could be metabolized by *Bacteroides* species. Although NSV may result from a mixed infection with *G. vaginalis* and certain anaerobic bacteria, the necessity for the presence of both types of micro-organism, and the role that either or both play in the initiation and development of the disease process is unclear.

One approach to the study of the aetiology and pathogenesis of NSV is to attempt to develop an animal model of the disease which would allow investigations of the relative roles of various micro-organisms. This report describes our attempts to infect the vaginal tract of three species of primate with *G. vaginalis* which was undertaken as a preliminary step in the possible development of an animal model of NSV. In particular, evidence was sought for the persistence of *G. vaginalis* organisms in the genital tract and the development of clue cells, as well as the development of humoral and local antibodies and changes in the levels of succinate and lactate in vaginal washings. Additionally, the anaerobic vaginal flora was studied to determine the species present.

Materials and methods

Animals. Twelve adult female pig-tailed macaques (*Macaca nemestrina*), six adult female tamarins (*Saguinus labiatus*) and four young female chimpanzees (*Pan troglodytes*) were used. The animals were cared for as described previously (Furr *et al.* 1976).

Gardnerella vaginalis. Four strains of *G. vaginalis* designated 584, 614, 812 and 958 were used. They were isolated from vaginal swabs obtained from women with non-specific vaginitis who attended the Praed Street Clinic, Paddington, London. The organisms were identified as *G. vaginalis* because they were Gram-variable rods which were oxidase and catalase negative, showed β-haemolysis on human but not horse blood agar, and fermented starch, maltose and dextrose (Ison *et al.* 1982).

Inoculation of animals. The inocula that the animals received in three experiments are shown in Table 1. In experiments numbers 1 and 2, organisms of strains 584 and 614 that had been subcultured only once were grown for 24 h in Peptone–Starch–Dextrose (PSD) broth containing 10% horse serum, washed twice and then resuspended in phosphate-buffered saline (PBS) to form the inocula. Organisms of strains 812 and 958, in contrast, had been subcultured many times before this study. To produce the inocula for experiment number 3, organisms were cultured on Bordet–Gengou plates for 2 days and the resulting growth was scraped off with a loop and resuspended in thioglycollate–glycerol broth (TGGB).

In each experiment the animals were

Table 1. Inoculation of animals with *Gardnerella vaginalis*

Experiment number	Animal	Inoculum	Number of animals
1	Pig-tailed macaque	Strain 584 (5×10^6 c.f.u.)	4
	Pig-tailed macaque	PBS	2
	Tamarin	Strain 584 (3×10^6 c.f.u.)	4
	Tamarin	PBS	2
2	Pig-tailed macaque	Strain 584 (1×10^7 c.f.u.)	7
	Pig-tailed macaque	Strain 614 (1×10^7 c.f.u.)	3
	Pig-tailed macaque	PBS	2
3	Chimpanzee	Strain 812 (5×10^7 c.f.u.)	2
	Chimpanzee	Strain 958 (1×10^8 c.f.u.)	1
	Chimpanzee	TGGB	1

c.f.u. Colony-forming units; PBS Phosphate-buffered saline; TGGB Thyoglycollate–glycerol broth.

anaesthetized with ketamine and inoculated intravaginally with a suspension of *G. vaginalis*, using either a sterile catheter or an Eppendorf pipette with sterile disposable tips. The pig-tailed macaques and chimpanzees were inoculated with 0.5 ml of bacterial suspension, while the tamarins received 0.3 ml. With some animals there was slight leakage of inoculum from the vagina. In each experiment, animals serving as controls were inoculated with PBS or TGGB not containing bacteria (Table 1).

Isolation of bacteria. To isolate *G. vaginalis*, a cotton-tipped swab was inserted into the vagina and rubbed against the vaginal wall. The swab was then rolled on a plate of double-layer human blood agar supplemented with gentamicin, nalidixic acid and amphotericin B (Ison *et al.* 1982). The inoculated area of the plate was spread with a loop and the plate was then incubated at 37°C in an atmosphere of 5% CO_2 in air for 48 h. Small colonies showing β-haemolysis were screened as described above.

In the studies with pig-tailed macaques, anaerobic bacteria were isolated by plating vaginal swabs on enriched blood agar (reinforced clostridial agar, Oxoid, 56 g/l; Liver Digest, Oxoid 10 g/l) and on a selective blood–agar medium (the medium described above supplemented with kanamycin and vancomycin). The plates were incubated in an anaerobic cabinet (Don Whitley) at 37°C for 5 days, and the resulting bacterial colonies were identified primarily by their inability to grow in air and their susceptibility to metronidazole (5 μg/ml). The organisms were identified at the species level by criteria described in the Virginia Polytechnic Insti-

tute manual. Attempts were not made to study the anaerobic vaginal flora of the tamarins or chimpanzees.

Vaginal smears. After taking swabs for isolation of bacteria, a further vaginal swab was smeared on a clean microscope slide. Smears were Gram-stained and examined microscopically for the presence of bacteria and 'clue cells'.

Vaginal pH. Vaginal pH values were measured using pH paper (May and Baker Ltd). On some occasions the pH paper was inserted into the vagina using artery forceps, while on other occasions a cotton-tipped swab was inserted into the vagina and then rolled on the surface of a small strip of paper.

Vaginal secretions. Vaginal washings were collected from pig-tailed macaques by introducing 1 ml of PBS into each animal's vagina with either an Eppendorf pipette, or a 1-ml syringe. The washings were centrifuged, the supernatant fluids being stored at $-20°$ until required for further testing, and the pellets were smeared on glass slides and Gram-stained.

Gas–liquid chromatography (GLC). The analysis of vaginal washings for non-volatile fatty acids was performed as described for human vaginal washings (Ison *et al.* 1983).

Antibody detection. An enzyme-linked immunosorbent assay was used to test for the presence of anti-*G. vaginalis* antibodies in sera and vaginal washings from macaques and sera from chimpanzees. The antigen was prepared from a 24-h broth culture of *G. vaginalis* by collecting the bacteria by centrifugation, washing in PBS and suspending in carbonate–bicarbonate buffer pH 9.6 at an optical density of 0.5 (λ 540 nm). Antigen (100 μl) was then added to the wells of microtitre plates (Linbro, Flow Laboratories) and left at room temperature for 30 min. The plates were washed three times with PBS containing 0.5% v/v tween 20, and the diluted sera or washings to be tested were added to the wells and incubated at 30°C for 1 h. The plates were then washed and peroxidase-labelled anti-human IgG or IgA was added and incubated for 2 h at 30°C. Preliminary studies indicated that the anti-human IgG and IgA cross-reacted with macaque immunoglobulins (C. Ison, unpublished observation). The level of antibody was determined by the addition of ortho-phenylene diamine in phosphate–citrate buffer pH 5.0 (0.4 mg/ml) and 4 μl H_2O_2 (3%). After incubation for 30 min the reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4 and the absorbance was read at 492 nm.

Results

Isolation of G. vaginalis from primates

Pig-tailed macaques. Before inoculation, each of the 12 pig-tailed macaques was swabbed either two or three times to detect the presence of indigenous *G. vaginalis* organisms. They were not isolated, however, from any of the pre-inoculation samples.

In the first experiment (Table 2) four animals received 5×10^6 colony-forming units (c.f.u.) of strain 584. *G. vaginalis* was isolated from two animals for at least 11 days after inoculation and from the other two animals for at least 17 and 33 days respectively. *G. vaginalis* was not recovered from either of the control animals inoculated with PBS.

In the second experiment a further six animals were inoculated with *G. vaginalis* (Table 2). Of three animals inoculated with 1×10^7 c.f.u. of strain 584, one was colonized for 11 days, while the other two were colonized for at least 39 days. Each of three animals given 1×10^7 c.f.u. of strain 614 were colonized for at least 39 days. Additionally, the four animals colonized in the first experiment were re-challenged with 1×10^7 c.f.u. of strain 584 and all but one (no. 25) became colonized, this time for at least 32 days.

Table 2. Isolation of *Gardnerella vaginalis* from the genital tract of pig-tailed macaques

Experiment number	Animal number	Inoculum	Isolation of *G. vaginalis* on indicated number of days after inoculation 4–5	11	17–18	25	32–33	39	47
1	16	584	+*	+	+	NT	−	−	−
	20	584	±	±	−	NT	±	−	−
	21	584	+	+	−	NT	−	−	−
	25	584	+	+	+	NT	−	−	−
2	18	584	+	+	+	±	+	+	NT
	19	584	+	±	−	−	−	−	NT
	23	584	+	+	−	±	±	+	NT
	6	614	+	−	+	+	+	+	NT
	22	614	+	+	+	−	−	+	NT
	24	614	+	±	±	+	−	+	NT
	16†	584	−	±	+	+	+	NT	NT
	20†	584	+	+	±	+	+	NT	NT
	21†	584	±	+	−	+	+	NT	NT
	25†	584	−	−	−	−	−	−	NT
	5	PBS	−	−	+	−	−	+	NT
	17	PBS	−	−	−	−	−	−	NT

* +, moderate to heavy growth; ±, scanty growth; −, no growth.
† Animals rechallenged.
NT, Not tested; PBS, phosphate-buffered saline.

On day 11 of the second experiment, all the animals were swabbed to detect the presence of *G. vaginalis* and vaginal washings obtained at this time were also tested for *G. vaginalis*. It was found that the washings from one control animal (no. 5) contained *G. vaginalis* (Table 2), although organisms had not been recovered from the vaginal swab. The most likely explanation is that an aerosol was generated when a washing from an infected animal was drawn into the Eppendorf tip. The aerosol contaminated the barrel of the pipette, which resulted in contamination of the fluid used to wash the vagina of animal no. 5, despite a new tip being used. When animal no. 5 was swabbed a week later (day 18, Table 2), *G. vaginalis* was recovered which indicated that cross-contamination from a colonized animal had occurred during the washing procedure. Although the next two swabs obtained from animal no. 5 were negative, a subsequent swab taken on day 39 of the experiment was positive.

Tamarins. Four tamarins were each inoculated with approximately 3×10^6 c.f.u. of strain 584, and two tamarins were inoculated with an equal volume of PBS (Table 1). The tamarins were inoculated immediately after four pig-tailed macaques had been inoculated in experiment no. 2, samples from the same bacterial suspension being used to provide the inocula for the two groups of animals. Nevertheless, swabs taken from each of the six tamarins before inoculation, and 5 and 11 days after inoculation, failed to yield *G. vaginalis*.

Chimpanzees. Two chimpanzees were each inoculated with 5×10^7 c.f.u. of strain 812. One chimpanzee was inoculated with

1×10^8 c.f.u. of strain 958 and one received TGGB. All four animals were swabbed 7 and 14 days after inoculation, but *G. vaginalis* was not recovered.

Vaginal smears

Vaginal smears were obtained from the pig-tailed macaques and chimpanzees but not from the tamarins. In general, Gram-stained smears contained squamous epithelial cells with a mixed bacterial flora. Occasionally, smears obtained from pig-tailed macaques showed some Gram-variable bacilli but they were not present in large numbers and cells covered with coccobacilli (clue cells) were not detected.

Vaginal pH

The vaginal pH of the pig-tailed macaques was determined on multiple occasions and was found to vary, usually within the range 6.0–7.0, although on occasions, pH values as high as 8.0 or 9.0 were recorded. The vaginal pH of each chimpanzee was determined on two occasions 1 week apart and was found to be between 5.5 and 6.0, while the vaginal pH of the tamarins, determined on one occasion only, was 7.0.

Isolation of anaerobic bacteria from pig-tailed macaques

Six of the pig-tailed macaques were sampled for anaerobes on five occasions, and six were sampled on two occasions. A total of 15 species of anaerobic bacteria was isolated (Table 3), 10 species belonging to the genus *Bacteroides*, and five belonging to the genus *Fusobacterium*. The two most common micro-organisms, each isolated from 10 of the 12 animals, were *B. fragilis* and a *Bacteroides* organism which could not be identified at the species level. No anaerobic cocci were isolated.

Non-volatile fatty acids in vaginal washings from pig-tailed macaques

Vaginal washings were obtained from all 12 pig-tailed macaques 4 days before they were inoculated in experiment no. 2. Although four animals had been inoculated with *G.*

Table 3. Species of anaerobic bacteria isolated from the lower genital tract of female pig-tailed macaques

Species	Animals from which organism was isolated
Bacteroides oralis	5
Bacteroides melaninogenicus sub. sp. *intermedius*	5,17,21
Bacteroides fragilis	5,6,16,17,19,20,21,22,23,25
Bacteroides ochraceus	5,18
Bacteroides ruminocola sub. sp. *ruminicola*	21,24
Bacteroides capillosus	21,22
Bacteroides eggerthii	20
Bacteroides amylophilus	22
Bacteroides nodussus	24
Fusobacterium gondiaformans	20,21
Fusobacterium nucleatum	20,22
Fusobacterium russii	20,21
Fusobacterium mortiferum	23
Fusiform sp	17,19
Unidentified anaerobic Gram-negative bacilli	5,6,16,17,19,20,21,23,24,25

vaginalis in the first experiment, they did not appear to be colonized at the time the washings were obtained since the organisms were not recovered from vaginal swabs taken on three successive occasions from each animal over the course of about 2 weeks. GLC analysis of the 12 washings for non-volatile fatty acids showed that nine of them had S/L ratios of $\geqslant 0.4$ (range 0.4–42.0), a value which has been suggested as diagnostic of NSV in humans (Spiegel *et al.* 1980), while only three washings had S/L ratios of <0.4 (0.06–0.33). Although a second set of washings was collected 11 days after inoculation they were not analysed by GLC in view of the high S/L ratios obtained at the start of the experiment.

Antibody studies

Serum samples were collected from the pig-tailed macaques and chimpanzees before and at least 2 weeks after inoculation. Additionally, vaginal washings were obtained from the macaques 11 days after inoculation. Samples were tested for IgG and IgA antibodies against the appropriate strain of *G. vaginalis*, but there was no significant increase in the titres of antibodies found.

Discussion

The results show that pig-tailed macaques are susceptible to vaginal colonization with *G. vaginalis*, each of 10 inoculated animals remaining culture positive for 11 to 39 days. Their susceptibility was emphasized by the fact that one of two uninoculated control animals also became transiently colonized, probably as the result of cross-contamination from an infected animal during the collection of vaginal washings. In contrast, four female tamarins inoculated with a strain of *G. vaginalis* that colonized each of four macaques in the same experiment, failed to become colonized. The size of inocula used was similar for macaques and tamarins as was the vaginal pH of both species. No tamarins were available for a repeat experiment. We must conclude, however, that there is a considerable inter-species variation in susceptibility.

Three chimpanzees inoculated with either of two strains of *G. vaginalis* also failed to become colonized. However, in contrast to the experiments with macaques and tamarins, where strains of *G. vaginalis* sub-cultured only once after isolation were used, the chimpanzees were inoculated with organisms that had been subcultured many times *in vitro*. In view of the known propensity of bacteria to decrease in virulence when cultured *in vitro* (Smith 1972) the failure to colonize chimpanzees may have been due either to their innate resistance or to a decreased ability of the organisms to colonize the genital tract.

The rationale for inoculating primates with *G. vaginalis* was to determine whether infected animals developed signs similar to those seen in women with *Gardnerella*-associated NSV. This proved difficult to evaluate, however, because all of the animals exhibited one or more of the characteristic signs of NSV before inoculation with *G. vaginalis*. For example, whereas the pH value for the normal adult premenopausal human vagina is 4.5 or less, and increases in cases of NSV (Spiegel *et al* 1980; Taylor *et al.* 1982), the vaginal pH value for all the animals was 6.0 or greater. Similarly, while the S/L ratio in vaginal washings from women without vaginitis is $\leqslant 0.4$ and increases to $\geqslant 0.4$ in cases of NSV, the S/L ratio for vaginal washings taken from macaques not infected with *G. vaginalis* was frequently $\geqslant 0.4$. The difference between the pH value and biochemical composition of human and macaque vaginal fluids is most likely due to the different composition of the vaginal flora. For example, lactobacilli, which constitute the predominant component of the vaginal flora of healthy human adults, were neither seen in Gram-stained vaginal smears nor isolated from vaginal swabs obtained from pig-tailed macaques (comparable data were not available for the tamarins or chimpanzees). An additional difference was the presence of *B. fragilis*, which was isolated, often repeatedly,

from 10 of the 12 macaques, but which is not isolated usually from the human vagina (Spiegel *et al.* 1980; Taylor *et al.* 1982).

Although the biochemistry and microbiology of the lower genital tract of female macaques is different from that of humans, it may be feasible to manipulate the conditions in the macaque vagina so that they resemble those seen in the healthy human vagina. For example, it may be possible to lower the vaginal pH of macaques by infecting with lactobacilli, assuming that the vaginal mucosal cells contain glycogen as a source of fermentable carbohydrate. A study of the biochemical changes that occur after vaginal colonization of such animals with *G. vaginalis* might be more useful for evaluating the role of this micro-organism in causing NSV. In the meantime, the susceptibility of untreated pig-tailed macaques to vaginal colonization with *G. vaginalis* may nonetheless prove of use in studying factors that influence the adhesion of *G. vaginalis* to vaginal epithelial tissue *in vivo*, and the subsequent interaction with the vaginal mucosa.

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