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Bacterial vaginosis in pregnancy: distribution of bacterial species in different gram-stain categories of the vaginal flora

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Vaginal swabs for microbiological culture were taken from 174 pregnant women whose vaginal flora had been evaluated by Gram's stain; 50 had grade III flora (bacterial vaginosis, BV), 50 grade II (intermediate), 41 had vaginal flora graded as abnormal which then reverted to grade I (revertants) and 33 had normal flora (controls). The aim was to determine whether bacterial species isolated from women with grade II flora differed from those with grade III flora. Isolation of *Lactobacillus* spp. decreased from grade I to grade III and that of other aerobic and anaerobic bacterial species increased. There was little difference in the species isolated from women with grade II and grade III flora, but there was a distinct order in which organisms in different species increased in numbers. The vaginal flora of revertants was intermediate between that of healthy controls and those with grade II flora. Coagulase-negative *Staphylococcus* spp. were isolated from a greater number of revertants than grade I controls but the incidence did not increase in grade II or grade III. *Bifidobacterium* spp. were isolated from a greater number of revertants than grade I controls and increased further in grade II and grade III. However, *Gardnerella vaginalis* and *Mycoplasma hominis* were isolated from a much larger number of women with grade III flora than the other groups. The conclusion is that grade II is a transitional phase between grade I and grade III and that some organisms such as *G. vaginalis* and *M. hominis* only reach large numbers in the late stage. The sequence of appearance of the various bacterial species may be a result of the pathophysiological alteration of the vaginal ecosystem associated with BV.

Introduction

The normal vaginal bacterial flora of healthy premenopausal women consists predominantly of *Lactobacillus* spp. [1]. These are believed to play a protective role in guarding the urogenital tract against infection by pathogens [2]. In bacterial vaginosis (BV), a condition characterised by a raised vaginal pH and milky discharge, the normal vaginal flora is replaced by a mixed flora of aerobic, anaerobic and micro-aerophilic species [1]. Although BV is common, in the past it has been regarded as a harmless abnormality [3, 4]. However, it is now considered to be associated with a variety of genital tract infections and complications including pelvic inflammatory disease, post-

caesarean delivery endometritis, post-hysterectomy vaginal cuff infection, post-abortal sepsis, premature rupture of membranes and preterm labour and delivery [4–10]. In a recent study of pregnant women, those who had BV on screening in early pregnancy were five times more likely to have a preterm delivery or second trimester spontaneous abortion than those women who did not have BV [11].

BV may be diagnosed on clinical grounds if three of the following four criteria are fulfilled [12] (composite criteria): the presence of a homogeneous grey-white discharge; a vaginal pH >4.5; vaginal epithelial cells covered with adherent bacteria ('clue' cells) in a 'wet mount' preparation; and the release of volatile amines by the addition of potassium hydroxide 5–10% to a small amount of vaginal fluid (the 'amine odour test'). The vaginal flora may also be assessed by Gram's stain of a smear prepared from a high vaginal swab [13–15]. Three categories may be recognised: grade I – normal, comprising predominantly lactobacillus

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morphotypes; grade II – intermediate, in which lactobacilli are reduced and mixed with other bacterial morphotypes; grade III – abnormal, with few or no lactobacillus morphotypes and with greatly increased numbers of other morphotypes, an appearance consistent with BV when diagnosed by clinical criteria. In a recent study by this group, the vaginal flora of women attending an antenatal clinic was graded in this way and an association was found between abnormal flora and an increased incidence of preterm delivery and late miscarriage [11]. A similar association between abnormal flora and preterm labour has been found by others [7, 16, 17] but not between BV and late miscarriage. There was also some indication in this study (unpublished observation) that preterm labour was most closely associated with grade III flora, whereas late miscarriage was associated with grade II flora. The current study was undertaken to determine by microbiological culture whether the bacterial species isolated from women with grade II and grade III flora are different or whether grade II simply represents a microbial continuum in the change from grade I to grade III and *vice versa*.

Materials and methods

Study population

Women attending the antenatal clinic at Northwick Park Hospital for the first time, at between 12 and 16 weeks and occasionally up to 20 weeks' gestation, were examined for evidence of BV as part of their routine antenatal examination. The diagnosis of BV was based on Gram's stain of vaginal secretion, as described previously [11].

The gram-stained slides were read after the clinic session. Women diagnosed as having abnormal flora (grade II or grade III) were asked to return to the clinic within 3 weeks to 1 month of their initial examination where they were examined again for evidence of abnormal flora by a Gram's stain of vaginal secretion. At this stage the women were also examined for clinical evidence of BV by the method described by Amsel *et al.* [12] (composite criteria). Women whose Gram's stain was still abnormal (grade II or grade III) were invited to enrol in a double-blind, placebo-controlled trial of a topically applied antibiotic cream (clindamycin 2%) for the prevention of BV-induced preterm delivery and early miscarriage. At the same time, high vaginal swabs were taken for full microbiological examination. Swabs were also collected from women who were described as 'revertants'. These women had an abnormal Gram's stain result on their first visit but, on returning to the clinic, their abnormal vaginal flora was found to have resolved spontaneously with a Gram's stain scored as grade I. In addition, there was no clinical evidence of BV as judged by composite criteria tests. Samples were also taken from women who had normal grade I

flora on initial and subsequent screening and clinically had no evidence of BV, and thus served as controls.

In this report, microbiological data are presented for 50 women with grade III flora and 50 women with grade II flora, randomly selected from the total population of women attending the antenatal clinic between January 1993 and March 1995, together with data from 41 revertants and 33 controls from the same population. Samples from a further 96 randomly selected women with BV were tested for *Mycoplasma hominis* and *Ureaplasma urealyticum* and samples from 50 of these women were also tested for *Chlamydia trachomatis*.

Sampling

The following swab specimens were obtained from the women. An endocervical swab for the detection of *C. trachomatis* was placed in sucrose-phosphate transport medium (Syva). An endocervical swab for the isolation of *Neisseria gonorrhoeae* was plated directly on to Modified New York City Medium (Oxoid). Plates were placed in a micro-aerophilic atmosphere (candle jar) and transported to the routine microbiology laboratory. They were then incubated in CO₂ 6% at 37°C and examined after incubation for 24 and 48 h. A high vaginal swab (HVS) for full microbiological culture was placed in bacteriological transport medium (tryptone 1%, yeast extract 0.5%, glucose 0.1% and cysteine hydrochloride 0.1% in sterile distilled water) and was agitated and squeezed thoroughly to dislodge its contents in the medium. The swab was then discarded and the transport medium was stored at –70°C within 1 h of inoculation and cultured (by IJR) within 6 months of storage. A HVS for the detection of *M. hominis* and *U. urealyticum* was placed in mycoplasmal transport medium [18], agitated and squeezed into the medium and stored as described above. A HVS was collected for the detection of motile *Trichomonas vaginalis* by microscopic examination of a saline wet mount preparation.

Processing of samples and detection of organisms

C. trachomatis was detected by an enzyme immunoassay (MicroTrak, Syva), according to the manufacturer's instructions. For bacteriological examination of the samples, 0.1 ml of the transport medium was plated on to each of the following media which were incubated at 37°C as follows: Columbia Agar (Oxoid) containing defibrinated horse blood 7%, incubated aerobically for 48 h, in a CO₂ (6%) incubator for 48 h and anaerobically (N₂ 80%, CO₂ 10%, H₂ 10%) for 5 days; Rogosa Agar (Oxoid) incubated micro-aerophilically and anaerobically for the isolation of lactobacilli; Brain Heart Infusion Agar (Oxoid), to which was added vancomycin 7.5 µg/ml, paromomycin 100 µg/ml (Parke Davis and Co.) and laked horse blood 10%, for the isolation of anaerobic gram-negative rods.

Different colony types were recorded and subjected to semi-quantitation as follows: few = 1–30 colonies at inoculum site; + = heavy growth at inoculum site and ≤10 colonies at first streak; ++ = heavy growth at inoculum site and first streak and ≤10 colonies at second streak; +++ = heavy growth at inoculum site and first streak and second streak and ≤10 colonies at third streak; ++++ = heavy growth at inoculum site and first, second and third streak and ≤10 colonies at fourth streak.

A colony of each type was subcultured on to Columbia blood agar. All organisms were identified presumptively by their morphology with Gram's stain. Colonies from anaerobic plates were subcultured in the same way and then incubated aerobically and anaerobically. Organisms that produced colonies that developed only anaerobically from two subcultures were considered to be anaerobic. All anaerobic organisms were identified presumptively by their morphology on Gram's stain. For coagulase production, colonies of aerobic, catalase-positive, gram-positive cocci were inoculated into nutrient broth containing plasma 10% and clotting was observed after 4 h.

For identification of *M. hominis* and *U. urealyticum*, 0.1 ml of transport medium was added to 0.9 ml of broth containing urea or arginine with phenol red as a pH indicator [18]. Further 10-fold dilutions were made up to 1 in 10⁸ for *M. hominis* and 1 in 10⁶ for *U. urealyticum* and the cultures were observed daily for 7 days for evidence of a colour change. The highest dilution (titre) at which a colour change occurred was considered to contain one colour-changing unit.

Results

The incidence of micro-organisms not recognised as part of the microbial flora of BV, namely *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*, is shown in Table 1. *N. gonorrhoeae* was not isolated from any of the four groups of women and trichomonads were detected rarely. Chlamydiae were detected most often in the BV group (12%). However, when the results of a further 50 women with BV from the same antenatal

population were included for analysis, the incidence of chlamydiae fell to 6%.

Distribution of micro-aerophilic and aerobic flora

The median number of micro-aerophilic and aerobic bacterial species isolated from each group was 2 (range 0–5) for grade I controls, 3 (range 0–5) for grade I revertants, 3 (range 0–5) for grade II and 4 (range 1–>5) for grade III.

Although there was a reduction in the incidence of lactobacilli from grade I to grade III flora, they were isolated from a substantial proportion of women with grade II and grade III flora (66% and 38%, respectively), sometimes in quite large numbers (Fig. 1). Conversely, lactobacilli were not isolated from 9% of women in the control group and from 22% in the revertant group, the latter reflecting perhaps in some instances a change back to clinical normality ahead of regaining a normal flora or, in other instances, a discrepancy between culture and Gram's stain results.

The bacterial flora pattern demonstrated by the revertant group was intermediate between that demonstrated by the healthy controls and those in the grade II flora group (Table 2). Due to initial difficulty with culture conditions, the results for *G. vaginalis* isolation were valid only for a proportion of specimens. Nevertheless, it was clear that *G. vaginalis* was isolated more frequently from women with grade III flora (72%) than from women in any other group (≤6%).

The proportion of women from whom various aerobic bacterial species were isolated increased from that for the grade I controls through the various grades to grade III (Table 2). This was seen particularly with *Corynebacterium* spp., coagulase-negative staphylococci and *Streptococcus* spp., although the former two were found in a large proportion of healthy grade I controls. *Candida* spp. were isolated from more women in the grade I and II groups and in larger numbers in the grade I revertants and grade II groups (data not shown) than the grade III group.

Of the 'enteropharyngeal' species, β-haemolytic streptococci were the only organisms isolated more

Table 1. Isolation of *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* from women with BV and healthy controls

Micro-organism	Isolation from women with Gram's stain of			
	Grade I (healthy) n = 33	Grade I (revertants) n = 41	Grade II (intermediate) n = 50	Grade III (BV) n = 50
<i>C. trachomatis</i>	0/12	1 (2%)	2 (4%)	6 (12%)
<i>N. gonorrhoeae</i>	0/8	0/30	0	0
<i>T. vaginalis</i>	1 (3%)	0	3 (6%)	0

Fig. 1. Distribution of *Lactobacillus* spp. in women with BV and healthy controls: **a**, grade I, healthy controls; **b**, grade I, revertants; **c**, grade II; **d**, grade III.

Table 2. Distribution of micro-aerophilic and aerobic flora in women with BV and healthy controls

Micro-organisms	Number (%) of women with the micro-organism in the following categories			
	Grade I (healthy controls) n = 33	Grade I (revertants) n = 41	Grade II (intermediate) n = 50	Grade III (BV) n = 50
Micro-aerophilic flora				
<i>Lactobacillus</i> spp.	30 (91%)	32 (78%)	33 (66%)	19 (38%)
<i>G. vaginalis</i>	1/21 (5%)	1/17 (6%)	2/38 (5%)	13/18 (72%)
Aerobic flora				
<i>Corynebacterium</i> spp.	12 (36%)	21 (51%)	28 (56%)	42 (84%)
Coagulase-negative	19 (58%)	32 (78%)	39 (78%)	37 (74%)
<i>Staphylococcus</i> spp.				
<i>Staph. aureus</i>	3 (9%)	3 (7%)	4 (8%)	7 (14%)
<i>Streptococcus</i> spp.*	3 (9%)	7 (17%)	18 (36%)	28 (56%)
<i>Streptococcus</i> spp.†	0	0	6 (12%)	2 (4%)
Gram-negative rods	0	3 (7%)	5 (10%)	6 (12%)
<i>Candida</i> spp.	7 (21%)	14 (34%)	13 (26%)	7 (14%)

* α -Haemolytic and non-haemolytic on Columbia blood agar.

† β -Haemolytic on Columbia blood agar.

frequently from women with grade II flora than from those with grade III flora (Table 2).

Distribution of anaerobic flora

The median number of anaerobic species isolated was 0 (range 0–3) for grade I controls, 0 (range 0–3) for grade I revertants, 2 (range 0–5) for grade II and 3 (range 1–5) for grade III.

Anaerobic bacteria were isolated far more often and in larger numbers (not shown) from women with grade II or grade III flora than from those with grade I flora (Table 3). Anaerobic gram-positive cocci and anaerobic gram-negative rods were isolated from a larger proportion of women in the grade III group than from those in the other groups. However, *Bifidobacterium* spp. were isolated from a much larger proportion of the revertants and those in the grade II group, compared with the isolation of anaerobic cocci and rods in these groups and from nearly 100% of women in the grade III group. Conversely, *M. hominis* was isolated from a much larger proportion of women with grade III flora compared with the revertants and those with grade II flora (Table 3). The proportion of women with *U. urealyticum* increased from grade I to grade III, but the difference was not as striking as that seen for *M. hominis*. *U. urealyticum* was isolated from a substantial proportion of the healthy women (42%) compared with the low isolation rate of *M. hominis* (6%)

A further 96 specimens from women with abnormal Gram's stain results (five of grade II and 91 of grade III) from the same antenatal population were tested for *U. urealyticum* and *M. hominis*. Analysis of larger numbers confirmed the difference in the distribution of these two micro-organisms seen previously. Thus, *U. urealyticum* was isolated from 49% of women with grade II flora and from 59% of those with grade III flora, whereas *M. hominis* was isolated from 14.5% of women with grade II flora and from 52% of those with grade III flora. The numbers of *M. hominis*

organisms in specimens from the four groups are shown in Fig. 2, large numbers occurring predominantly in women with grade III flora.

Sequence of changes in the flora

Fig. 3 illustrates the changes that might occur in the microbial flora of the vagina, culminating in BV. The increase in colonisation by coagulase-negative staphylococci occurred at an early stage (grade I revertants) but did not increase further. In contrast, other bacteria such as *Bifidobacterium* spp. appeared early (grade I revertants) and continued to increase in grades II and III, while other species, such as *G. vaginalis* and *M. hominis*, were only found in large numbers of patients in the late stage (grade III).

Discussion

The vaginal microbial flora in pregnancy has been studied previously in relation to preterm delivery, miscarriage and intra-uterine growth retardation [7, 8, 10, 11, 17, 19]. The current study, which to our knowledge is the most extensive and detailed in the UK of the microbiology of BV in pregnancy, was undertaken to determine whether differences exist between the bacterial species isolated from the vagina of pregnant women with grade II flora compared with those isolated from women with grade III flora. The recognition of a group of women defined as 'revertants' is novel and is not in the pattern of vaginal flora described by Hillier *et al.* [15]. Nevertheless, the results of the present study are consistent with those of these workers in demonstrating that the flora can switch from the abnormal state (graded as III) to the normal state (graded as I), passing through an intermediate stage (graded as II). This can occur rapidly, within 3–4 weeks, as the revertant group of 41 women observed contained four whose flora changed from grade III to grade I between clinic visits. The flora of the other 37 women changed from grade II to grade I.

Table 3. Distribution of *M. hominis*, *U. urealyticum* and anaerobic flora in women with BV and in healthy controls

Micro-organisms	Number (%) of women with the micro-organism in the following categories			
	Grade I (healthy controls) n = 33	Grade I (revertants) n = 41	Grade II (intermediate) n = 50	Grade III (BV) n = 50
<i>M. hominis</i>	2 (6%)	7 (17%)	7 (14%)	29 (58%)
<i>U. urealyticum</i>	14 (42%)	21 (51%)	25 (50%)	34 (68%)
Anaerobic flora				
Gram-positive cocci*	5 (15%)	7 (17%)	20 (40%)	37 (74%)
Gram-negative rods†	3 (9%)	10 (24%)	21 (42%)	33 (66%)
<i>Bifidobacterium</i> spp.	4 (12%)	17 (41%)	29 (58%)	47 (94%)
Other gram-positive rods‡	5 (15%)	6 (15%)	9 (18%)	24 (48%)

* *Peptococcus* spp., *Peptostreptococcus* spp.

† *Bacterioides* spp., *Prevotella* spp., *Fusobacterium* spp.

‡ *Mobiluncus* spp., *Propionibacterium* spp.

Fig. 2. Distribution of *M. hominis* in women with BV and healthy controls: **a**, grade I, healthy controls; **b**, grade I, revertants; **c**, grade II; **d**, grade III.

Although the type of organisms isolated from women with grade II and grade III flora may not be different, there does appear to be an order in which the different species appear. This is illustrated clearly in Fig. 3 and has not been highlighted by others. As expected, a large proportion of healthy women with grade I flora had lactobacilli in large numbers. Coagulase-negative staphylococci were often present, as were *Corynebacterium* spp. and *U. urealyticum*. In the revertant group, compared with women who had grade I flora, a much larger proportion carried coagulase-negative staphylococci and *Bifidobacterium* spp. in large numbers. The proportion of women with grade II flora from whom *Bifidobacterium* spp. were recovered was greater than that of the revertant group, as was the proportion of women with grade II flora carrying anaerobic gram-positive cocci and anaerobic gram-negative rods. In contrast, the proportion of women with coagulase-negative staphylococci in the two groups was similar. It was only in grade III that *G. vaginalis* and *M. hominis* were found in a large proportion of the

women. Like us, Hillier *et al.* [15] recorded a high incidence of *U. urealyticum* in the normal grade I group, an incidence which increased steadily through the intermediate to the BV group. However, unlike these workers, this study found that the incidence of *G. vaginalis* and *M. hominis* did not increase steadily, but the organisms were isolated almost uniquely in the grade III group.

It is clear from the results of this study that the initial disturbance in the vagina causes certain bacteria, such as coagulase-negative staphylococci, to appear first, in large numbers, followed by *Bifidobacterium* spp. However, it is then unclear whether rapid multiplication of these organisms provides an environment conducive to the multiplication of other bacterial species, such as anaerobic gram-negative rods and gram-positive cocci, and then finally *G. vaginalis* and *M. hominis*, or whether it is the alteration of the environment *per se* which causes this sequence of events to occur. Examination of physiological factors,

Fig. 3. Percentage incidence of various BV-associated bacterial species in different gram-stain categories of the vaginal flora. **a**, ●, *Lactobacillus* spp.; ▲, coagulase-negative *Staphylococcus* spp.; ■, *Candida* spp.; **b**, ●, *Bifidobacterium* spp.; ▲, anaerobic gram-positive cocci; ■, anaerobic gram-negative rods; **c**, ●, *G. vaginalis*; ▲, *M. hominis*; ■, *U. urealyticum*.

such as hormone and secreted immunoglobulin concentrations in the vagina, might help to distinguish between the two possibilities although, of course, they may not be mutually exclusive.

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