

## Selective Differential Human Blood Bilayer Media for Isolation of *Gardnerella (Haemophilus) vaginalis*

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New selective and differential human blood bilayer agar media with Tween 80 (HBT medium) or without Tween 80 (HB medium), developed for the isolation of *Gardnerella (Haemophilus) vaginalis*, permitted significantly higher *G. vaginalis* isolation rates than have been obtained for other media used for this purpose. HB medium consists of a basal layer of Columbia agar base containing colistin and naladixic acid with added amphotericin B and an overlayer of the same composition plus 5% human blood. HBT agar also contains Proteose Peptone No. 3 (Difco Laboratories) and Tween 80 in the basal layer and the overlayer. Both Tween 80 and the bilayer composition enhanced *G. vaginalis* production of human blood hemolysis, permitting detection of this organism even in the presence of heavy growth of other vaginal flora. The use of HB or HBT medium thus permitted the demonstration that *G. vaginalis* was present in vaginal fluid from a large percentage (up to 68%) of normal women. However, the concentration of *G. vaginalis* was found by semiquantitative analysis to be significantly higher in vaginal fluid from women with nonspecific vaginitis than in fluid from normal women.

Since Gardner and Dukes (7) first isolated *Gardnerella (Haemophilus) vaginalis* from women with nonspecific vaginitis (NSV) in 1955, there has been controversy over the role of this organism in NSV. Some investigators (6, 13) have noticed no difference in *G. vaginalis* prevalence between vaginal fluid of women with vaginitis and vaginal fluid of women without vaginitis, whereas others (1, 12, 14) have isolated *G. vaginalis* more often from women with NSV than from normal control women.

Variables affecting studies of the correlation of *G. vaginalis* with NSV may include criteria used to diagnose NSV, comparability of NSV patients with controls, media used for isolation of *G. vaginalis*, and criteria used for identification of this organism. Recent taxonomic studies (10, 15) have proposed placement of this organism in a new genus, *Gardnerella*, and have clarified the characteristics of this organism, making more-accurate identification possible.

Other media previously used by others for isolation of *G. vaginalis* have included chocolate agar (3, 14), peptone-starch dextrose (PSD) agar 1, 5, 8), sheep blood-beef infusion agar (13), V agar (11, 18), Columbia-colistin-naladixic acid (CNA) agar (8), Casman solid medium (6), and

starch agar (17, 18). Some of these media are not selective or differential; the recognition of *G. vaginalis* on PSD agar requires the use of a dissecting microscope (5). The present study concerns the development and evaluation of new selective differential media for improved *G. vaginalis* isolation.

### MATERIALS AND METHODS

**Collection of specimens.** Vaginal fluid was collected for culturing with calcium alginate swabs (Inolex, Glenwood, Ill.). The swabs were used to inoculate an area ca. 2.5 cm<sup>2</sup> in a corner of each medium to be tested. A wire loop was then flamed and used to cross-streak from this area into the first zone. The loop was again flamed and used to streak from the first zone into a second zone and from the second zone into a third zone.

The order in which the plates were inoculated was systematically varied. No more than two plates were inoculated with the same swab. The plates were held at 37°C in candle jars until they could be transported to the lab.

**Study population.** Vaginal specimens were collected for culturing from three sources: (i) consecutive students reporting to the Women's Health Clinic, University of Washington (i.e., all students reporting to the clinic during the study); (ii) consecutive women who had gonorrhoea and coincidentally were found to have clinical evidence of NSV, seen at the Sexually Transmitted Diseases Clinic at Harborview Medical Center, Seattle; and (iii) patients referred for treatment of NSV

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TABLE 1. Comparison of rates of isolation of *G. vaginalis* on HB and chocolate agar media: study of 60 college students and 60 patients referred for NSV treatment

Medium	No. of isolations/total no. of isolates (%)				
	College students cultured <sup>a</sup>		Patients referred for NSV treatment <sup>b</sup>		
	NSV	Normal <sup>c</sup>	Pretreatment	Posttreatment	
				Clinical failure	Clinical cure <sup>d</sup>
HB	11/12 (92)	18/48 (38)	17/17 (100)	16/20 (80)	14/23 (61)
Chocolate	11/12 (92)	4/48 (8)	16/17 (94)	14/20 (70)	5/23 (22)

<sup>a</sup> Cultured from January to July, 1979.

<sup>b</sup> Cultured from January, 1979, to February, 1980.

<sup>c</sup>  $P = 0.001$ .

<sup>d</sup>  $P = 0.016$ .

to the Harborview Medical Center Sexually Transmitted Diseases Clinic vaginitis study. The number of patients evaluated in each group is shown in Tables 1 through 4.

**Definition of NSV.** As previously described (16), patients were diagnosed on their initial visit as having NSV if they had a negative microscopic examination for yeasts and *Trichomonas vaginalis* and if two or more of the following abnormal findings were present: (i) the pH of vaginal fluid was  $>4.5$ , (ii) homogeneous vaginal discharge was present, (iii) clue cells were present in vaginal fluid, and (iv) fishy amine odor was released when vaginal fluid was mixed with 10% KOH. Women were asked to return 1, 2, and 6 weeks after initiation of treatment with metronidazole, ampicillin, or amoxicillin, administered by standardized protocol, and were classified as treatment failures if two or more of the above abnormal findings were still present (a pH of  $\leq 4.7$  was regarded as normal after treatment).

**Media.** H (human blood) medium consisted of approximately 21 ml of CNA agar (Columbia agar base containing colistin and nalidixic acid; BBL Microbiology Systems), with amphotericin B (2  $\mu\text{g/ml}$ ) and 5% human blood added after autoclaving was performed. HB (human blood bilayer) medium was composed of a basal layer of 7 ml of CNA agar base, with amphoteri-

cin B (2  $\mu\text{g/ml}$ ) added after autoclaving was performed, and a 14-ml overlay of the same composition plus 5% human blood. HBT medium was the same as HB medium except that 1% Proteose Peptone No. 3 (Difco Laboratories) was added to both layers before autoclaving was performed, and .0075% Tween 80 (BBL) was added to both layers after autoclaving was performed. Tween 80 used after its expiration date had a lowered pH and resulted in a shorter shelf life of the plates. We used freshly outdated human blood of any blood group obtained from the Puget Sound Blood Center, Seattle, Wash.

Chocolate agar was composed of GC agar base (BBL) plus 1% Isovitalax enrichment (BBL) and 5% heated "chocolatized" sheep blood. V agar (11) was composed of a single layer of 5% human blood added to Columbia agar base (BBL) and Proteose Peptone No. 3 (Difco). PSD agar has been described previously (4). For subculturing of *G. vaginalis* isolates, a human blood subculture medium was made from a single layer of Columbia agar base (BBL) containing 5% human blood. Medium used for sugar fermentation testing consisted of 1% Proteose Peptone No. 3 (Difco), 0.3% meat extract (BBL), 0.5% NaCl, and 1% Andrade indicator. This medium was adjusted to pH 7.3 with NaOH and autoclaved. To this base, the appropriate sugar was added to a final concentration of 1%.

TABLE 2. Comparison of beta-hemolysis produced by *G. vaginalis* on HB medium with that produced on three other media<sup>a</sup>

Medium	No. of beta-hemolytic colonies (proportion)			No. of cultures showing indicated clarity of hemolysis (proportion)		
	+	=	-	+	=	-
	<sup>b</sup>	<sup>c</sup>	<sup>d</sup>	<sup>e</sup>	<sup>f</sup>	<sup>g</sup>
H (single layer)		0.45	0.55		0.27	0.73
HB + Proteose Peptone No. 3		1.00		0.35	0.65	
HB + 0.01% Tween 80	0.18	0.82		0.69	0.31	

<sup>a</sup> Vaginal secretions from 21 college students and 4 women with gonorrhea, obtained from September to October, 1979, were cultured on all four media (see text for formulations). Of these 25, 9 had been diagnosed as having NSV. *G. vaginalis* was isolated from 21 patients, including all 9 with NSV.

<sup>b</sup> +, Proportion of colonies that were beta-hemolytic was greater than the proportion obtained on HB medium.

<sup>c</sup> =, Proportion of colonies that were beta-hemolytic was equal to the proportion obtained on HB medium.

<sup>d</sup> -, Proportion of colonies that were beta-hemolytic was less than the proportion obtained on HB medium.

<sup>e</sup> +, Proportion of cultures showing clarity of hemolysis greater than that obtained on HB medium.

<sup>f</sup> =, Proportion of cultures showing clarity of hemolysis equal to that obtained on HB medium.

<sup>g</sup> -, Proportion of cultures showing clarity of hemolysis less than that obtained on HB medium.

TABLE 3. Comparison of rates of isolation of *G. vaginalis* on HB, HBT, chocolate, and V media: study of 35 college students and 12 women with gonorrhea and NSV

Medium	No. of isolations/total no. of women (%)		
	College students <sup>a</sup>		Women with gonorrhea and NSV <sup>a</sup>
	NSV	Normal	
HB	6/6 (100)	17/29 (59)	10/12 (83)
HBT	6/6 (100)	20/29 (69)	10/12 (83)
V	5/6 (83)	8/29 (28)	6/12 (50)
Chocolate	5/6 (83)	13/29 (45)	4/12 (33)

<sup>a</sup> Cultured from January to February, 1980.

**Evaluation of media.** All plates were incubated in 5% CO<sub>2</sub> at 37°C and were read at 48 h and rechecked at 72 h before being discarded. Technologists examining the media were blinded to the clinical status of the patient from whom each culture was taken. When chocolate agar was used, growth on the plates was examined and interpreted separately from growth on plates containing human blood.

HB, HBT, chocolate, and V agar media were used for up to 1 month after preparation. Initial experiments showed that the human blood hemolysis produced by *G. vaginalis* on BBL Columbia agar base was superior to that obtained on the same base obtained from Difco. Thereafter, only BBL CNA agar and Columbia agar base were used.

Growth was quantitated as follows: 1+, <10 colonies in the first zone of inoculation; 2+, >10 colonies in the first zone and <10 colonies in the second zone; 3+, >10 colonies in the second zone and <10 colonies in the third zone; and 4+, >10 colonies in the third zone.

**Identification of *G. vaginalis*.** Colonies of *G. vaginalis* were of necessity identified on different media by different criteria. Colonies on chocolate agar medium were identified as pinpoint colonies appearing in 48 or 72 h; they produced no surrounding green discoloration of the agar. Colonies on human blood agar media were identified as small white colonies which produced beta-hemolysis after 48 or 72 h of incubation. Single colonies isolated on chocolate and human blood agar media were transferred to chocolate agar medium and human blood subculture medium, respectively. These subculture media were used to prepare inocula for the other test media. Bacteria identified as *G. vaginalis* were always pleomorphic, gram-variable, catalase-negative rods, and they fermented starch but not mannitol. In addition, we determined the following by the methods described by Piot et al. (15a): the zone of inhibition around a 50-μg metronidazole disk on PSD agar medium, the zone of inhibition around a disk containing 1 mg of bile on PSD agar medium, and the

zones of inhibition around a 150-μg nitrofurantoin disk and around a 1-mg triple sulfonamide disk on human blood subculture agar medium. These tests were used only for identification, since conditions used were not standardized for antibiotic susceptibility testing. Colonies selected for subculturing and for confirmation as *G. vaginalis* were submitted to all of the above-named confirmation tests, whether or not they produced beta-hemolysis on the single-layer human blood subculture agar. All isolates identified as *G. vaginalis* were later tested for human blood hemolysis on HB medium and for production of green discoloration of chocolate agar.

**Statistical analyses.** The Fisher exact test was used for all comparisons.

## RESULTS

**Development of HB medium.** CNA agar was used by Goldberg and Washington (8) as a selective medium for isolation of *G. vaginalis*. To make this medium differential as well as selective for *G. vaginalis*, we added human blood to CNA agar medium. Human blood was used because Greenwood et al. (9) reported that 96% of *G. vaginalis* isolates produce beta-hemolysis of human blood, whereas none are beta-hemolytic for sheep blood. Our initial experiments showed that *G. vaginalis* consistently produced more easily detected beta-hemolysis on plates poured with relatively shallow agar layers, but shallow agar plates had a short shelf life and became excessively dried during incubation. Bilayer plates containing a thin overlayer of human blood (HB medium) proved more satisfactory. Amphotericin B was added to inhibit yeasts, and the resulting medium was designated HB medium. This medium was then compared with chocolate agar plates, which had

TABLE 4. Semiquantitation of *G. vaginalis* on HB medium in relation to clinical diagnosis of NSV in 204 college women (cultured from February to August, 1979)

Diagnosis (n)	No. (%) of women yielding growth of <i>G. vaginalis</i> at indicated quantity of growth				
	No growth	1+	2+	3+	4+
NSV (44)	3 (6.8)	0 (0)	1 (2.3)	10 (22.7)	30 (68.2)
No NSV (160)	96 (60.0)	7 (4.4)	15 (9.4)	19 (11.9)	23 (14.4)

been previously used in our lab for NSV patient cultures.

Isolation of *G. vaginalis* on chocolate and HB media was evaluated by using vaginal secretions from 60 college students and 60 patients referred for NSV treatment (Table 1). Among the 60 students, 12 were found to have NSV, and *G. vaginalis* was isolated from 11 of the 12 (92%) on both HB and chocolate agar media. However, among the remaining students who had normal vaginal examinations, *G. vaginalis* was isolated from 18 of 48 (38%) on HB medium and only 4 of 48 (8%) on chocolate agar ( $P = .001$ ).

Among specimens from women referred for treatment of NSV, *G. vaginalis* was nearly always isolated on both HB and chocolate agar media before treatment. After treatment with metronidazole, ampicillin, or amoxicillin, *G. vaginalis* was usually isolated on both media in specimens from 20 women with persistent or recurrent NSV (clinical failures), but the organism was isolated significantly more often on HB medium than on chocolate agar in specimens from 23 women who were clinically cured (14 of 23 versus 5 of 23;  $P = .016$ ).

**Evaluation of modifications of HB medium.** We next evaluated several modifications of HB medium. The rate of isolation of *G. vaginalis*, colony size, and rate and extent of beta-hemolysis after 48 h of incubation on HB medium were compared with the results obtained for media which differed from HB medium by just one variable: H medium (single layer rather than bilayer), HB medium plus Proteose Peptone No. 3, and HB medium plus 0.01% Tween 80. Samples from 21 students and 4 women with gonorrhea were collected and streaked on each of the plates as described above. After incubation at 37°C in 5% CO<sub>2</sub> for 48 h, *G. vaginalis* colonies on all media were examined and compared. *G. vaginalis* was isolated on H medium from 56% of the women, on HB medium from 80%, on H medium plus Proteose Peptone No. 3 from 76%, and on HB medium plus 0.01% Tween 80 from 84%.

Beta-hemolysis on HB (bilayer) medium was superior to that on H (single-layer) medium, and the addition of Tween 80 or Proteose Peptone No. 3 to both layers of the HB agar further enhanced beta-hemolysis produced by *G. vaginalis* colonies (Table 2). The *G. vaginalis* isolation rate was higher on the bilayer media (76 to 84%) than on single-layer H agar (56%), because the more-apparent hemolysis on bilayer media facilitated detection of *G. vaginalis*.

The colony size on HB medium containing Proteose Peptone No. 3 or Tween 80 was generally equal to or larger than the colony size on HB medium alone (data not shown).

Further and separate experiments showed

that HB supplemented with both Tween 80 and Proteose Peptone No. 3 (HBT medium) gave better colony size and hemolysis than did HB medium alone. The concentration of Tween 80 in HBT medium was lowered from .01 to .0075% in subsequent experiments, since the shelf life of the plates was longer with this lower concentration.

**Clinical comparison of HB and HBT media with other media commonly used for isolation of *G. vaginalis*.** The rate of isolation of *G. vaginalis* on HB and HBT media was then compared with the rate of isolation on chocolate and V agar media. Two groups of women were cultured: a third group of 35 college students and a group of 12 women who had both gonorrhea and NSV (Table 3). *G. vaginalis* was isolated on all four media from specimens of most students found to have NSV, but among specimens of college students who had normal examinations, *G. vaginalis* was isolated most often on HBT medium and least often on V agar ( $P = .007$  for HBT versus V agar). The rate of isolation of *G. vaginalis* from vaginal specimens from college women was higher on both HB and chocolate agar media during this study (Table 3) than during the initial evaluation (Table 1), probably because the research laboratory technologists became more skilled at detection of *G. vaginalis* as a result of working with the improved media.

Among the 12 specimens from women with gonorrhea and NSV, *G. vaginalis* was also isolated more often on HB and HBT media than on V or chocolate agar media, and the isolation rate was significantly higher on HB and HBT media than on chocolate agar ( $P = .04$ ). This unusually low rate of *G. vaginalis* isolation on chocolate agar in specimens from women with NSV may have been due to *Neisseria gonorrhoeae* growth interfering with recognition of *G. vaginalis* on this medium.

During all comparisons of these media, in only one case was *G. vaginalis* isolated only on chocolate agar but not on HB agar (this strain subsequently was found to be beta-hemolytic on HBT medium), and in no case was *G. vaginalis* isolated on V agar but not on HB (or HBT) medium. HB and HBT media completely inhibited gram-negative rods and yeasts and partially inhibited staphylococci and diphtheroids.

The rates of isolation of *G. vaginalis* on plates incubated for 48 and 72 h were also evaluated. The percentages of cultures that were positive for *G. vaginalis* at 72 but not 48 h were as follows: chocolate agar, 5 of 50 (10%); HB, 7 of 64 (11%); and HBT, 1 of 67 (1%).

Figure 1 shows the typical appearance of these media inoculated with vaginal specimens from women with and without NSV 48 h after incubation in 5% CO<sub>2</sub>.

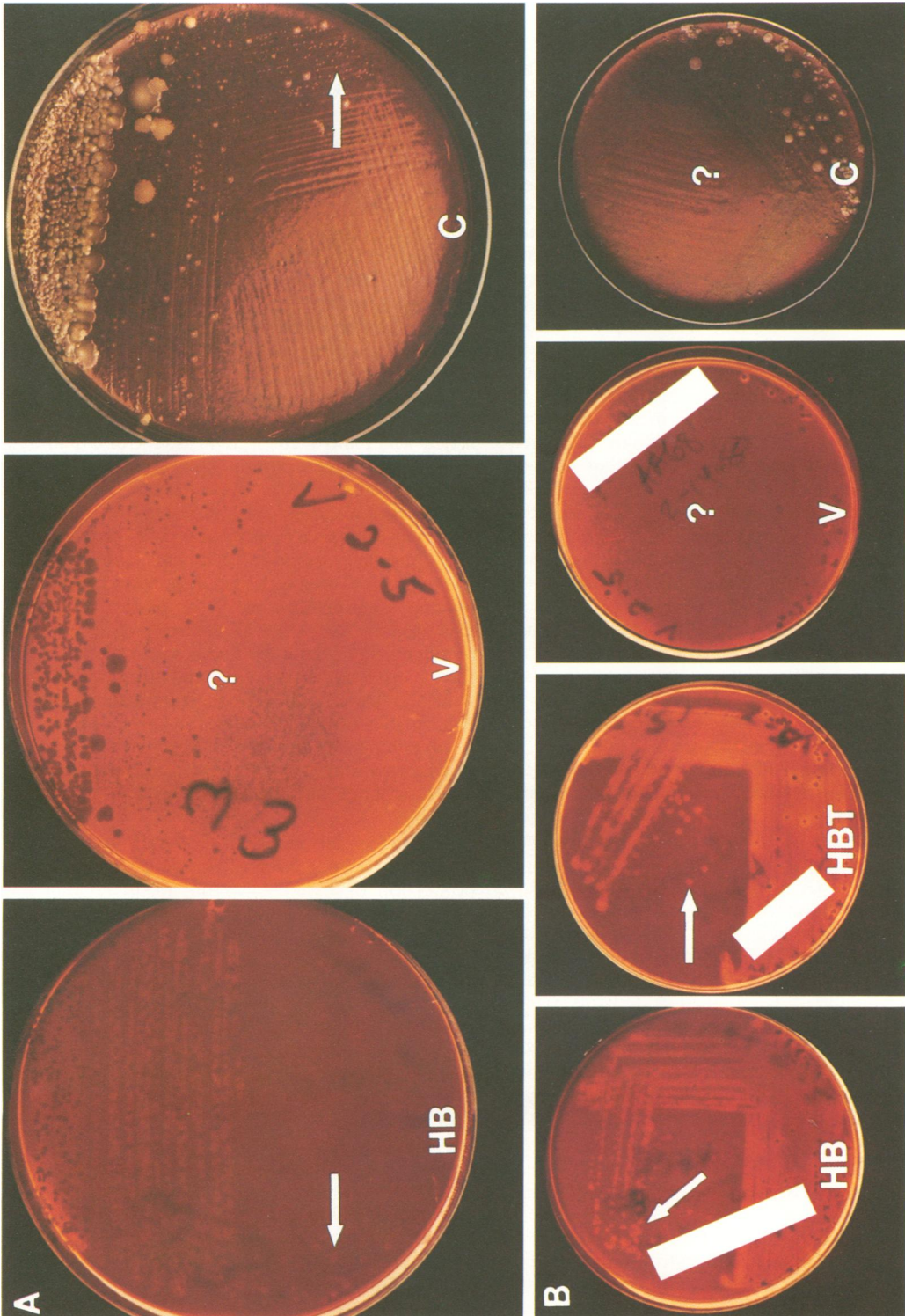


FIG. 1. Examples of vaginal cultures of patients before and after treatment for NSV. (A) Results of cultures from a woman with NSV. *G. vaginalis* is readily distinguishable on HB medium but not on V agar medium. On chocolate agar (C), many other small, nonpigmented colonies resembling *G. vaginalis* were also present which produced no green discoloration of the medium. (B) Results of cultures from a woman treated for NSV. No clinical manifestations of NSV remained. *G. vaginalis* was present on HB medium, hemolysis was enhanced on HBT medium, but *G. vaginalis* was not discernible on chocolate or V agar medium.



**Accuracy of presumptive identification of *G. vaginalis* colonies picked for subculturing from primary isolation media.** To analyze the accuracy of presumptive identification of *G. vaginalis* colonies on various media, we compared the number of colonies picked for subculturing from the primary isolation plates with the number of colonies confirmed as *G. vaginalis* by our identification scheme. Of 51 colonies selected from chocolate plates, 88% (45 of 51) were confirmed to be *G. vaginalis*. Most isolates not confirmed to be *G. vaginalis* were eliminated by Gram stain results; these isolates also had aberrant results for starch fermentation, nitrofurantoin sensitivity, bile sensitivity, or combinations of the three. One isolate had a typical Gram stain but was sensitive to sulfonamides. This is consistent with the "*H. vaginalis*-like strains" described by Bailey et al. (2). Presumptive identification of *G. vaginalis* on HB and HBT media was more accurate; 51 of 51 beta-hemolytic colonies picked from HB and 27 of 28 (96%) beta-hemolytic colonies picked from HBT medium were confirmed to be *G. vaginalis*. Of 212 isolates tested, 7 (3.3%) showed no zone of inhibition around the metronidazole disk, but since other tests were compatible, they were identified as *G. vaginalis*.

Of 28 strains of *G. vaginalis* isolated on chocolate agar, 86% (24 of 28) were beta-hemolytic on HB, and 100% were beta-hemolytic on HBT ( $P = .11$ ). None of 67 strains of *G. vaginalis* isolated on HB or HBT medium produced green discoloration of chocolate agar.

**Semiquantitation of number of colonies of *G. vaginalis* on HB medium: comparison of primary cultures from women with and without NSV.** For specimens from 204 women attending the University of Washington's Women's Health Clinic, the growth of *G. vaginalis* on HB medium was analyzed semiquantitatively in relation to the diagnosis of NSV (Table 4). *G. vaginalis* was isolated in 3+ or 4+ amounts from 91% of the specimens from women with NSV but only 26% of the specimens from normal controls ( $P < .001$ ). Among specimens from which *G. vaginalis* was isolated on HB medium, growth was 4+ in 30 of 41 (73%) specimens from women with NSV versus 23 of 64 (36%) specimens from women without NSV ( $P < .001$ ).

## DISCUSSION

In this study, we chose to compare HB and HBT agar media with V agar and chocolate agar media because the latter two media are commonly used in clinical laboratories today. Another commonly used medium, PSD, was not tested, because of the time-consuming necessity of using a dissecting microscope to identify *G. vaginalis* on PSD (5). Starch agar, a modification

of PSD, has given isolation rates comparable to that found for V agar in one study (18) and therefore was not compared here.

HB, HBT, V, and chocolate agar media all proved sensitive for the isolation of *G. vaginalis* from specimens of women referred for the treatment of NSV, probably because of the high concentration of *G. vaginalis* present in the vaginal secretions of women with NSV. However, for specimens from women who had been treated for NSV and specimens from normal women, HB and HBT media were more sensitive than V or chocolate agar for *G. vaginalis* isolation. HBT medium containing supplemental Proteose Peptone No. 3 and Tween 80 gave the greatest degree of beta-hemolysis around colonies of *G. vaginalis* and showed the best growth of this organism after 48 h of incubation. Tween 80 caused spontaneous hemolysis during storage or incubation in early experiments, a problem which was controlled by adjustment of pH and Tween 80 concentration. The HB and HBT media were not only more sensitive for the detection of *G. vaginalis* but also more specific for the presumptive identification of *G. vaginalis* upon primary isolation. Clinical laboratories may wish to evaluate the need for tests confirming the identification of *G. vaginalis* isolates from vaginal fluid, since all but one isolate which produced beta-hemolysis and typical colonies on HB or HBT agar were subsequently confirmed to be *G. vaginalis*. Experienced clinical microbiologists may only need to use the Gram stain test to presumptively identify colonies which have the morphology typical of *G. vaginalis* and which produce beta-hemolysis on HB or HBT medium.

The increased sensitivity of HB and HBT media allowed detection of *G. vaginalis* in a high proportion of specimens from normal women and permitted the demonstration that among women with vaginal *G. vaginalis* carriage, the concentration of *G. vaginalis* is higher among those who have NSV. These observations illustrate that the pathogenesis of NSV and, specifically, the role of *G. vaginalis* in this syndrome are not fully understood. Recent evidence indicates that anaerobes may participate with *G. vaginalis* in the production of this syndrome (16). These observations pose problems for those who believe that the causal relationship between *G. vaginalis* and NSV is a simple one. In the past, studies using less-sensitive chocolate agar medium have led some investigators (14) to observe that *G. vaginalis* was present in nearly all women with NSV and in only a small proportion of women with normal vaginal exams: such studies have led to the point of view that *G. vaginalis* may be a conventional sexually transmitted pathogen producing NSV in the ma-

jority of infected women. The present study shows that the majority of women carrying *G. vaginalis* do not have NSV. These observations have interesting implications for clinical microbiologists, who are often called upon to provide diagnostic testing for NSV. Isolation of *G. vaginalis*, even in concentrations of 3+ or 4+, did not accurately predict the presence of clinical findings of NSV (Table 4). For example, among the 204 college women studied, the prevalence of NSV was 22%. In these college women, even the isolation of 3+ or 4+ growth of *G. vaginalis* was predictive of clinical findings of NSV in only 40 of 82 (49%). Thus, the positive predictive value of isolation of 3+ or 4+ growth of *G. vaginalis* was only 49%. On the other hand, the negative predictive value of a negative *G. vaginalis* culture (the probability that a patient with a negative culture did not have clinical manifestations of NSV) was 96 of 99 (97%).

In the future, additional clinical diagnostic information may be obtained from the use of HB or HBT medium. For example, we did not, in this study, systematically quantitate the growth of lactobacilli on HB or HBT medium. It is possible that the combination of 3+ or 4+ *G. vaginalis* growth and the absent or diminished growth of lactobacilli on HB or HBT medium has a higher predictive value for the diagnosis of NSV than the observation of increased *G. vaginalis* growth alone. HB or HBT agar medium should also prove useful for the study both of the epidemiology of *G. vaginalis* in men and women and the treatment of NSV. These media could be used to determine whether the persistence of *G. vaginalis* in women who appear to be clinically cured after treatment correlates with subsequent relapse of NSV. These media may also be used for the study of the relationship of *G. vaginalis* to other syndromes, such as puerperal and neonatal infections.

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