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Identification of *Gardnerella* (*Haemophilus*) *vaginalis*

PETER PIOT,[1]* EDDY VAN DYCK,[1] PATRICIA A. TOTTEN,[2] AND KING K. HOLMES[2,3]

Laboratory of Microbiology, Institute of Tropical Medicine, 2000 Antwerp, Belgium,[1] U.S. Public Health Service Hospital, Seattle, Washington 98114,[2] and Division of Infectious Diseases, Department of Medicine, University of Washington, Seattle, Washington 98105[3]

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Different tests for the identification of *Gardnerella* (*Haemophilus*) *vaginalis* and for its differentiation from catalase-negative unclassified coryneforms from the vagina were evaluated on over 200 bacterial strains, with special emphasis on optimal test conditions. A presumptive identification of *G. vaginalis* in the clinical laboratory can be made on the basis of colonial morphology, clear beta-hemolysis with diffuse edges on human blood bilayer-Tween agar, a negative catalase test, and typical cell morphology in the Gram stain. This procedure will correctly identify 90 to 98% of suspect colonies of *G. vaginalis* with human blood bilayer-Tween agar as primary isolation medium. Useful additional reactions for the confirmation of *G. vaginalis* include positive hippurate and starch hydrolysis, positive α-glucosidase but negative β-glucosidase tests, the production of acid from glucose and maltose but not from mannitol, and susceptibility to disks containing metronidazole, nitrofurantoin, sulfonamides, and bile.

The major problem in the identification of *Gardnerella* (*Haemophilus*) *vaginalis* in the clinical laboratory is its differentiation from other catalase-negative, small gram-variable rods. The latter occur commonly in vaginal specimens from women with and without vaginitis, but have been poorly characterized. The classic identification protocol of Dunkelberg et al. (4) is primarily based on the recognition of colonial morphology on peptone-starch-dextrose (PSD) agar with a dissection microscope. Bailey et al. (1) recommended the use of disk susceptibility testing for metronidazole and sulfonamides, in conjunction with fermentation tests. The most straightforward scheme for the presumptive identification of *G. vaginalis* was given by Greenwood and Pickett (5), who stressed the importance of beta-hemolysis on V agar (human blood) but not on sheep blood agar.

Different tests for the identification of *G. vaginalis* and for its differentiation from vaginal coryneforms were evaluated in this study, with special emphasis on the optimal test conditions. Recommendations for the presumptive identification of *G. vaginalis* in clinical laboratories and for the confirmation of its identity in reference laboratories are made.

MATERIALS AND METHODS

Strains. The following *G. vaginalis* strains were included in the study: strain ATCC 14018 (NCTC 10287) was obtained from the National Collection of Type Cultures, London, England; strains VF 16.1, VF 54.2, and 66.2 were supplied by I. Phillips, London, England; 12 strains of *G. vaginalis* were supplied by I. Kallings, Stockholm, Sweden; 56 strains were obtained as clinical isolates from the Division of Infectious Diseases, U.S. Public Health Service Hospital, Seattle, Wash.; 80 strains were obtained as clinical isolates from the clinical microbiology laboratory, Institute of Tropical Medicine, Antwerp, Belgium; and 23 strains were isolated in the Department of Microbiology, University of Nairobi, Nairobi, Kenya.

The following strains were considered to be *G. vaginalis*: strains belonging to cluster 8 in a previously reported numerical taxonomic study (9), strains showing a high degree (≥72%) of DNA-DNA homology with *G. vaginalis* ATCC 14018, and strains received as *G. vaginalis* from the respective clinical laboratories.

Unclassified coryneform organisms (UCOs) were catalase-negative bacteria isolated from the vagina and morphologically resembling *G. vaginalis*, but which did not meet the criteria for *G. vaginalis* mentioned above. Eight strains of unclassified vaginal coryneforms were supplied by I. Phillips, London, England, 19 were described elsewhere in a taxonomic study (9), and another 43 strains were obtained from the clinical microbiology laboratory, Institute of Tropical Medicine, Antwerp, Belgium.

The following streptococci were tested when the hippurate hydrolysis test was evaluated: *Streptococcus agalactiae* ITG 1, ITG 71, and ITG 1924; *S. bovis* ITG 1263 and ITG 1636; *S. equisimilis* NCTC 5371 and ITG 1620; *S. faecalis* NCIB 6782; *S. pneumoniae* ITG 1211, ITG 1218, and ITG 1220; *S. pyogenes* Cummins S6; and *S. sanguis* NCTC 7864.

Media. Bacterial strains were grown on H agar consisting of Columbia agar base (BBL Microbiology Systems) with 5% human blood. Human blood bilayer-Tween 80 agar (HBT agar) has been described elsewhere (12), and PSD agar was prepared as described by Dunkelberg and McVeigh (2).

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Tests. The hippurate hydrolysis test used was a modification of the procedure of Harvey (6). The substrate solution was composed of 1 g of sodium hippurate, 73.2 ml of 0.067 M KH_2PO_4, and 26.8 ml of 0.067 M $Na_2HPO_4 \cdot 2H_2O$ (final pH 6.4). This solution was filter sterilized, dispensed in 0.5-ml volumes into Parafilm (American Can Co.)-sealed tubes, and stored at −20°C. To test the influence of pH on hippurate hydrolysis, phosphate buffer solutions with 1% sodium hippurate with pH ranging between 5.2 and 8.0 were used. The tubes were inoculated with a loopful (2-mm-diameter loop) of bacteria from an overnight culture on H agar. After incubation at 37°C (water bath) for 2 h, 0.2 ml of a 3.5% ninhydrin solution in equal parts of acetone and butanol was added. Reactions were read with the naked eye and in a Bausch & Lomb photometer at 580 nm after 5 min at 37°C.

The presence of α- and β-glucosidase was tested in a 0.1% solution of 4-nitrophenyl-α-D-glucopyranoside (E. Merck AG) and 4-nitrophenyl-β-D-glucopyranoside (E. Merck AG), respectively, in 0.067 M Sörensen's phosphate buffer, pH 8.0. Solutions were filter sterilized and stored at 4°C. Tubes containing 0.5 ml of the substrate solution were inoculated as for hippurate hydrolysis. The tubes were incubated at 37°C in a water bath and read after 4 and 18 h for the appearance of a yellow color.

The production of acid from carbohydrates was determined in a modification of the medium of Dunkelberg et al. (3). The medium consisted of: proteose peptone no. 3 (Difco Laboratories), 1.5 g;-$Na_2HPO_4 \cdot 2H_2O$, 0.207 g;$NaH_2PO_4 \cdot H_2O$, 0.038 g; phenol red, 0.005%; distilled water, 95 ml; and carbohydrate, 1 g. After filter sterilization, 5 ml of sterile horse serum was added. The pH was adjusted to 7.5 with 5 N NaOH. The medium was stored at 4°C for a maximum of 4 weeks. Tubes containing 0.5 ml of fermentation medium were inoculated as above and incubated at 36°C in air. The tubes were examined daily for 5 days.

Beta-hemolysis was detected on HBT agar (Totten et al., submitted for publication) incubated in a 5% CO_2 atmosphere for 48 h.

Starch hydrolysis was determined on Mueller-Hinton agar (BBL) enriched with 5% (final concentration) sterile horse serum. Plates were inoculated and incubated anaerobically (GasPak system, BBL) at 36°C for 3 days and then flooded with iodine reagent (2% Lugol).

The lipase reaction was performed on an egg yolk medium as described in the *Anaerobe Laboratory Manual* (7), but the medium was enriched with 5% horse serum and 1% proteose peptone no. 3 (Difco). Plates were incubated anaerobically (GasPak system) at 36°C for 3 days.

In tests for inhibition by H_2O_2, bile, and antimicrobial agents, the following disks were used: metronidazole, 50 μg (Difco); nitrofurantoin, 50 and 150 μg (Difco); "triple sulfa," 1 mg (BBL); tobramycin, 10 μg (Difco); trimethoprim, 5 and 15 μg; pyronine tablet (1:1,600) (Key Scientific Products); bile, 10 μl of a 10% aqueous solution of oxgall (Difco); and H_2O_2, 10 μl of a 3% solution. The last two disks were prepared in the laboratory with BBL blank paper disks. Bacteria from 24-h cultures on H agar were suspended in 2 ml of sterile saline to the turbidity of McFarland standard no. 2. A cotton swab was used to inoculate PSD and H plates with the microorganisms. The disks were then added to the plates. The diameters of the inhibition zones around the disks were recorded after incubation for 48 h in a 5% CO_2 atmosphere at 36°C. Bile sensitivity was tested only on PSD agar since bile caused hemolysis of the blood in the H plates. Sensitivity to H_2O_2 was not tested on H medium since this medium contains human blood. Predictive values of a positive and of a negative test were calculated as described by Vecchio (13).

RESULTS

Standardization of tests. Before evaluating the usefulness of certain reactions, it was necessary to standardize and optimize test conditions.

Initially, the hippurate hydrolysis reaction was not completely reproducible, and the meaning of delayed or weak reactions was unknown. Therefore, 14 strains of streptococci with well-known reactions in this test (4 positive and 10 hippurate negative) were used to study the influence of pH on the test result. The range of pH levels tested was between 5.2 and 8.0, with intervals of 0.4. At pH 5.2 to 6.0, 2 of 10 hippurate-negative streptococci (*S. pyogenes* Cummins S6 and *S. pneumoniae* ITG 1220) yielded a blue-purple color, but the color was not as deep as that observed with the hippurate-positive strains. Weaker false-positive reactions occurred at pH 6.8 to 7.6, whereas at pH levels above 7.2 false-negative reactions were found. The optimal pH of the substrate solution, giving no false-negative or false-positive reactions and giving clear-cut reactions was 6.4. The optimal time for reading after addition of the ninhydrin solution was approximately 5 min. Shaking the test solution occasionally produced false-positive reactions.

Initially, acid formation from carbohydrates was often weak, delayed, and irregular for the same strain when Dunkelberg's fermentation medium (3) enriched with 5% horse serum and adjusted to pH 7.3 was used. Subsequently, it was found that use of 24-h cultures instead of 48-h cultures to inoculate the tests nearly doubled the number of clear-cut positive reactions and eliminated many delayed positive reactions (detailed results not shown). The carbohydrate medium was also weakly buffered, and the pH was increased from that used by Dunkelberg to 7.5. This resulted in more clear-cut reactions, with almost all tubes showing either a yellow or a deep red color. When these test conditions were rigorously followed, fermentation tests were highly reproducible. Positive reactions with maltose and starch usually occurred within 1 h (range, 0.5 to 2 h); positive reactions with glucose usually occurred within 4 h (range, 0.5 to 12 h). The production of acid from other carbo-

TABLE 1. Features of 175 strains of *G. vaginalis* and of 70 selected catalase-negative coryneforms from the vagina

Test	*G. vaginalis*		UCOs	
	Test result	% Positive	Test result	% Positive
Beta-hemolysis (human blood-bilayer)	+	99	V[a]	40
Beta-hemolysis (human blood-single layer)	+	88	−	10
Alpha-hemolysis (human blood-bilayer)	−	0	V	37
Hippurate hydrolysis	+	90	V	58
Starch hydrolysis	+	100	V	71
Lipase	V	64	V	24
Growth on Mueller-Hinton agar	+	100	V	58
α-Glucosidase	+	100	+	94
β-Glucosidase	−	0	V	57
β-Galactosidase	V	45	V	55
Acid from:				
Arabinose	V	38	−	13
Glucose	+	100	+	100
Maltose	+	100	+	98
Mannitol	−	0	V	20
Starch	+	100	+	98
Sucrose	+	85	V	81
Trehalose	V	72	V	52
Xylose	V	44	V	22

[a] Variable.

hydrates was slower, occasionally taking as long as 5 days.

Up to 10% of *G. vaginalis* strains yielded poor growth on the starch hydrolysis medium when incubation was in a 10% CO_2 atmosphere. All of these strains grew heavily on this medium when it was incubated anaerobically (GasPak), and all showed a positive starch hydrolysis reaction.

Features of *G. vaginalis*. Table 1 summarizes the results from 17 tests for 175 strains of *G. vaginalis* and 70 UCOs from the vagina.

With the exception of one isolate which apparently lost its hemolytic activity on subculture, all *G. vaginalis* strains were beta-hemolytic on HBT agar. Approximately half of the UCOs showed beta-hemolysis on HBT medium. However, hemolysis by these strains was nearly always weaker and the zone was of smaller diameter than for *Gardnerella*-type hemolysis. The latter zone was 1 to 2 mm wide around isolated colonies on HBT agar after 48 h of incubation and was clear with diffuse edges. Beta-hemolysis was demonstrated on single-layer human blood medium with 88% of *G. vaginalis* strains, but with only 10% of the UCOs.

Two-thirds of the UCOs showed colonies on HBT or H agar that were smaller and slower growing than those of *G. vaginalis*. Moreover, approximately half of the unidentified coryneforms were pleomorphic, fairly thick, and predominantly gram-positive rods to coccobacilli. These were readily differentiated from *G. vaginalis* cells, which were thin, gram-negative or gram-variable, short rods. Ten percent of the UCOs showed the classic appearance of lactobacilli in the Gram stain, i.e., regular gram-positive rods, often in chains. The cell morphology of the remaining 40% of UCOs was indistinguishable from that of *G. vaginalis* cells.

All *Gardnerella* strains hydrolyzed starch, produced acid from glucose, maltose, and starch, grew on Mueller-Hinton agar, and were α-glucosidase positive. None fermented mannitol, was β-glucosidase positive, or showed alpha-hemolysis on human blood agar.

Diameters of inhibition zones with different antimicrobial agents and other inhibitory agents are given in Table 2. Zone diameters were easier to read on H agar than on PSD agar. This was a result of the better growth of *G. vaginalis* on blood agar and the difference in color of the white *G. vaginalis* colonies on the red H medium. However, zone sizes were generally smaller on blood agar. With the exception of 50- and 150-μg nitrofurantoin disks on PSD agar, there was always some overlapping in zone sizes between those for *G. vaginalis* and UCOs. The differentiation of *G. vaginalis* and UCOs was better on H agar with sulfonamides. With metronidazole, PSD agar yielded better differentiation, since all *G. vaginalis* strains gave some inhibition zone on PSD agar but not on H agar.

Table 3 shows the sensitivity, specificity, and predictive value of a positive test and the predictive value of a negative test for several tests and combinations of tests for the identification of *G.*

TABLE 2. Zone sizes observed in disk inhibition tests with 46 *G. vaginalis* strains and 25 catalase-negative UCOs[a]

Disk	PSD agar		H agar	
	G. vaginalis	UCOs	*G. vaginalis*	UCOs
Bile, 10%	20 (14–28)	NZ (NZ–24)	NT	NT
H_2O_2, 3%	55 (47–74)	37 (15–75)	NT	NT
Metronidazole, 50 μg	44 (12–55)	NZ (NZ–52)	21 (NZ–40)	NZ (NZ–40)
Nitrofurantoin, 50 μg	64 (50–80)[b]	NZ (NZ–44)[c]	53 (43–70)	12 (NZ–50)
Nitrofurantoin, 150 μg	69 (50–80)[b]	17 (NZ–42)[c]	58 (50–74)	22 (NZ–54)
Pyronine, 1:1,600	21 (12–30)	NZ (NZ–26)	NZ (NZ–14)	NZ (NZ–12)
Sulfonamide, 1 mg	NZ (NZ–16)	NZ (NZ–66)	NZ	NZ (NZ–60)
Tobramycin, 10 g	NT	NT	NZ	11 (NZ–29)
Trimethoprim, 5 μg	34 (18–50)	NZ (NZ–35)	32 (NZ–43)	NZ (NZ–42)
Trimethoprim, 15 μg	44 (21–64)	NZ (NZ–46)	37 (25–49)	NZ (NZ–35)

[a] Results are given as the median (and range) of diameters of inhibition zones in millimeters. NZ, No inhibition zone around disk; NT, not tested.
[b] Forty-four strains tested.
[c] Twenty-three strains tested.

vaginalis in the bacterial population studied (175 strains of *G. vaginalis* and 70 strains of UCOs, giving a "prevalence" of *G. vaginalis* of 71%).

DISCUSSION

Many discrepancies in test results for *G. vaginalis* are presumably due to differences in test methods. Reproducibility of some of the tests used was unsatisfactory until rigorously standardized procedures were used. It was found that inoculation of tests with overnight cultures generally gave quicker, stronger, and more reproducible test results than those obtained with 48-h cultures. A large inoculum equally contributed to the elimination of doubtful or slow reactions.

The test results obtained here are in good agreement with those found by Greenwood and Pickett (5) and in our taxonomic study on a more limited number of strains (9). However, more positive fermentation reactions were obtained for sucrose, trehalose, and xylose than in our previous study or in Greenwood and Pickett's survey (5).

The enzymatic *o*-nitrophenol tests for the detection of α- and β-glucosidase gave unequivocal results within 2 h, usually within 30 min. They were highly reproducible, could be performed in small volumes, and gave mutually exclusive results with *G. vaginalis*. Additional advantages were that the test result was not influenced by the growth rate of the organism in the test medium and that the substrate solution was stable at 4°C in the dark for at least 3 months.

The disk inhibition tests described above were simple, cheap, and reproducible, but required at least 24 h before inhibition zones were measurable. Inhibition zones were not always easily observed on PSD agar after 24 h because of inadequate growth. The most reliable and reproducible results were obtained when the zones were read at 48 h.

The problem in the clinical laboratory and in the reference laboratory is to differentiate *G. vaginalis* from unclassified catalase-negative coryneforms. In this study, no single test was absolutely specific for *G. vaginalis*, but this is very rarely the case in the identification of any bacterial species.

TABLE 3. Sensitivity, specificity, and predictive value of a positive test and predictive value of a negative test for several combinations of tests for the identification of *G. vaginalis* in the bacterial population studied (175 strains of *G. vaginalis*, 70 strains of unidentified vaginal coryneforms)

Test					Sensitivity (%)	Specificity (%)	Predictive value positive (%)	Predictive value negative (%)
Beta-hemolysis	α-Glucosidase	β-Glucosidase	Hippurate hydrolysis	Starch hydrolysis				
+					99	59	86	98
+	+	−			99	85	95	98
+			+		89	76	90	73
+	+	−	+		88	91	98	76
+	+	−	−		11	90	73	29
+	+	−	+	+	88	99	99	91
+	+	−	−	+	11	96	86	32

Table 3 gives a minimal estimate of the predictive value of certain combinations of tests for the identification of *G. vaginalis*. The predictive value of a positive test is the likelihood that a strain yielding a positive test or combination of tests actually belongs to the species *G. vaginalis* (13). The frequency (or "prevalence") of *G. vaginalis* among other similar bacterial species influences the predictive value of a positive test heavily. The data from this study are biased towards low predictive values because of an unusually high frequency of "problem" strains. These were included to reflect the situation in a reference laboratory which receives only selected, difficult cases. A better idea of the true predictive value for beta-hemolysis on HBT agar is given by the identification of 80 isolates picked from HBT agar inoculated with specimens from 58 women with nonspecific vaginitis and 22 women without vaginitis. With the identification scheme, 78 of 80 were confirmed as *G. vaginalis*, giving a predictive value for a positive test of 97.5% for *Gardnerella*-type beta-hemolysis on HBT agar. The high predictive value of beta-hemolysis on HBT agar as a primary isolation medium may be due partly to the fact that a single colony of a UCO is less likely to be beta-hemolytic on a primary isolation plate of HBT agar than it is on a subculture on HBT plates.

On the basis of these results, the following recommendations for the identification of *G. vaginalis* are made. In the clinical laboratory a presumptive identification can be made based on colonial morphology, clear beta-hemolysis with diffuse edges on HBT agar, a negative catalase test (with the slide test), and the typical cell morphology in a Gram-stained smear. This procedure will correctly identify *G. vaginalis* in 90 to 97% of cases. Absence of hemolysis on sheep blood or nongreening of chocolate agar could be added to this scheme until the clinical microbiologist feels confident in ruling out lactobacilli based on Gram stain. Fermentation reactions do not differentiate *G. vaginalis* from other organisms. Larger clinical laboratories or laboratories with a research interest in genital infections may wish to perform additional tests. A maximal discriminative value (up to 99%) is given by the combination of the tests for α- and β-glucosidase and for hippurate and starch hydrolysis (Table 3). With the exception of the last reaction, all results can be read within 2 h. Alternatively, one can use the disk inhibition tests, which discriminate equally well and are simple to perform but require at least 24 h.

Unclassified vaginal coryneforms frequently present the following features not listed in Table 1: some are strongly gram positive and are thicker than *G. vaginalis* cells, often showing club-shaped swellings, and most are only weakly hemolytic on HBT agar and form colonies that are smaller or more irregular than those of *G. vaginalis*. On the basis of these morphological features, an experienced laboratory technician can discard most of the non-*G. vaginalis* isolates.

TABLE 4. Recommended tests for the confirmation of *G. vaginalis* (not listed: Gram stain, colonial morphology, type of beta-hemolysis on human blood)

Test	*G. vaginalis*	UCOs
Catalase	−	−
Beta-hemolysis on HBT agar	+	−
Hippurate hydrolysis	+	Variable
Starch hydrolysis	+	Variable
α-Glucosidase	+	+
β-Glucosidase	−	Variable
Acid from:		
Maltose	+	+
Mannitol	−	Variable
Inhibition zone with:		
Metronidazole, 50 μg (PSD agar)	Any zone	No zone
Nitrofurantoin, 150 μg (H agar)	>45 mm	<45 mm
Sulfonamide, 1 mg (H agar)	No zone	Variable
Bile, 10% (PSD agar)	Any zone	No zone

A reference laboratory has fewer constraints of time and usually receives highly selected, problem strains. Useful reactions for the identification of *G. vaginalis* are listed in Table 4. Analysis of volatile and nonvolatile end products by gas-liquid chromatography can provide additional information, but does not necessarily differentiate *G. vaginalis* from bifidobacteria or the *Haemophilus vaginalis*-like organisms described by Bailey et al. (1). All three groups of organisms produce acetic and lactic acids and minor amounts of formic acid.

The recently proposed scheme of Greenwood and Pickett (5) is very similar to the one recommended here. Their scheme includes an oxidase test, which is negative for nearly all catalase-negative coryneforms resembling *G. vaginalis* and consequently can be omitted. The scheme of Bailey et al. (1) is also similar to ours but is based on the selection of colonies which ferment starch on starch agar (10). This feature is not sufficiently specific for *G. vaginalis*, as shown in Table 4. Other nonspecific tests include inhibition of *G. vaginalis* by 3% hydrogen peroxide (4) or by *S. pneumoniae* (8) and the absence of a tellurite reduction (11). However, they may be useful for the confirmation of *G. vaginalis* in a reference laboratory.

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