

Rapid Microbiochemical Method for Identification of *Gardnerella (Haemophilus) vaginalis*

DAVID C. T. YONG¹* AND J. STEPHEN THOMPSON²

Laboratory Services Branch, Ontario Ministry of Health, Windsor Regional Public Health Laboratory, Windsor,¹ and Peterborough Regional Public Health Laboratory, Peterborough,² Ontario, Canada

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A rapid biochemical method for the identification of *Gardnerella vaginalis* has been developed. The method is based on the fermentation of starch and raffinose and on the hydrolysis of hippurate. With this new procedure, identification was confirmed for 390 of 396 *G. vaginalis* isolates within 1 h after their inoculation into the three substrates.

Gardner and Dukes (5) proposed the name *Haemophilus vaginalis* for the catalase- and oxidase-negative, gram-negative bacilli which they isolated from the vaginal secretions of patients with nonspecific vaginitis and from the genitourinary tracts of their sexual contacts (6). Subsequent investigators, however, observed the organism to be pleomorphic and, under certain conditions, to stain either gram-variable or gram-positive. These observations, together with the fact that the organism could be cultured on media lacking X and V factors, led to the suggestion that it be placed in the genus *Corynebacterium* (14, 24). More recently, however, taxonomic studies by Greenwood and Pickett (10) and Piot et al. (18) showed that the organism is not related to *Haemophilus* or *Corynebacterium*, and the former group proposed that it be placed in a new genus, *Gardnerella*. Nomenclature for this organism remains unresolved, and in this paper we will use the name *Gardnerella vaginalis*.

Although *G. vaginalis* has been closely associated with vaginitis, nongonococcal urethritis, cystitis, and abortion (5, 13, 15, 17, 19), the report by some workers that the organism can be isolated from the vaginal tract of symptomless individuals has clouded the view of *G. vaginalis* as a pathogen (7, 12). However, as noted in a recent review by Gardner (4), the majority of the publications on clinical studies of *G. vaginalis* support the original observation that this organism is the cause of nonspecific vaginitis. Additionally, Spiegel et al. (22) have recently shown that *G. vaginalis* was the only organism consistently isolated from the vaginal secretions of patients with nonspecific vaginitis. Since most publications support *G. vaginalis* as a pathogen, it therefore follows that a simple and rapid laboratory procedure should be available to identify this organism.

Several approaches for the identification of *G. vaginalis* have been suggested (1-3, 8, 11, 16). Unfortunately, there is no agreement as to the number and selection of laboratory tests to be used as criteria for the definitive identification of this organism.

Some investigators have reported the fermentation of dextrose, maltose, starch, and lactose (2, 3, 11, 16) as a means of identifying *G. vaginalis*. Others (1, 9) have shown that this approach has drawbacks, since fermentation of dextrose and lactose is variable for *G. vaginalis*, and use of a biochemical scheme that requires fermentation of dextrose or lactose will not correctly identify all isolates. In addition, use of criteria based on the fermentation of dextrose, maltose, starch, and lactose will fail to differentiate *G. vaginalis* from other catalase- and oxidase-negative and gram-negative-staining *G. vaginalis*-like organisms, since *G. vaginalis*-like organisms and *G. vaginalis* will ferment maltose (1). Smith has shown that *G. vaginalis*-like organisms are raffinose positive, whereas *G. vaginalis* is raffinose negative, and therefore feels that this is a useful differential test. As an adjunct to biochemical tests, Greenwood and Pickett (9) have proposed that the production of beta-hemolysis on human blood agar (V agar) be used, in part, as an identifying characteristic of *G. vaginalis*.

The fact that most isolates of *G. vaginalis* have been reported to hydrolyze hippurate (9) and ferment starch (1, 9) but not to ferment raffinose (21) suggests that these tests would prove reliable as final identification criteria for *G. vaginalis*.

This report presents the results of a study in which a rapid micro-starch-hippurate-raffinose (RM-SHR) method for the definitive identification of *G. vaginalis* was used and is the first time that these three substrates have been evaluated

as a group to identify this organism. The principle of this method is adapted from a method previously described for the identification of *Neisseria gonorrhoeae* (23).

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MATERIALS AND METHODS

Cultures. A stock culture of *G. vaginalis* obtained from the Central Public Health Laboratory, Ontario Ministry of Health, Toronto, Canada, was used in this study as a reference strain.

A total of 423 organisms were tested, consisting of 396 strains of *G. vaginalis*, 21 *G. vaginalis*-like organisms isolated in our laboratories, and a further six strains of *G. vaginalis*-like organisms which were obtained from R. F. Smith, Public Health Laboratory, Contra Costa County Health Department, Martinez, Calif. The organisms isolated in our laboratories were identified as described below.

Isolation and identification of cultures used in this study. In one of our laboratories, the organisms were isolated and subcultured on Gonococcus base (Difco Laboratories) plus 10% sheep blood. In the other laboratory, the organisms were isolated on Columbia blood agar (Columbia agar base [GIBCO Diagnostics] plus 5% sheep blood).

Isolation media inoculated with vaginal swabs or cervical swabs or both were examined after 24 h of incubation at 37°C in a 5% CO₂ environment for the presence of tiny "dew drop" smooth transparent colonies. Preliminary tests for the presumptive identification of these isolates as *G. vaginalis* consisted of a Gram-stained smear and oxidase and catalase tests.

Isolates which were catalase- and oxidase-negative and were observed to be gram-negative bacilli were tested further by inoculation into cystine-Trypticase agar (CTA) medium (BBL Microbiology Systems) with the addition of dextrose, maltose, starch, or lactose as described by Shtibel and Toma (20).

Isolates presumptively identified by the above criteria were tested biochemically in parallel by the conventional method with CTA dextrose, maltose, starch, and lactose and by the RM-SHR technique. Organisms which were CTA positive in dextrose, maltose, and starch and negative in lactose were considered to be *G. vaginalis*. Organisms not demonstrating this profile were sent to the Reference Bacteriology Section of the Central Public Health Laboratory, Ontario Ministry of Health, for final identification. Organisms were identified as *G. vaginalis*-like if they morphologically resembled *G. vaginalis*, were gram-negative to gram-variable, were oxidase and catalase negative, and also exhibited one of the following patterns in the rapid micro-tests: (i) starch negative, hippurate positive or negative; (ii) raffinose positive; or (iii) starch positive hippurate negative.

Subsequently, each of the previously identified isolates was retested with RM-SHR media and CTA-

based dextrose, maltose, starch, and lactose media in the comparative study reported here.

RM-SHR media. The media for raffinose and starch fermentation tests were prepared as follows. Rapid micro-base consisted of Casamino Acids (Difco; certified), 2 g; L-cysteine-hydrochloride, 0.03 g; sodium sulfate (J. T. Baker Chemical Co.), 0.03 g; neopeptone (Difco), 2.5 g; phenol red, 0.01 g; and distilled water, 100 ml. For completed rapid micro-media, 2 g of raffinose (Difco) or 2 g of soluble starch (Fisher Scientific Co.) was added to 100 ml of base. The pH was adjusted to 7.20 to 7.25 with 1 N sodium hydroxide. The complete raffinose medium was sterilized by filtration through a 0.45- μ m membrane filter (Millipore Corp.). Starch medium was sterilized by autoclaving at 121°C for 10 min.

The medium for the hydrolysis of hippurate was prepared as follows. For hippurate solution, 0.25 g of sodium hippurate (Difco) was added to 25 ml of distilled water. The solution was sterilized by filtration through a 45- μ m membrane filter (Millipore Corp.). Microorganisms were inoculated into this substrate. For ninhydrin solution, 3.5 g of ninhydrin was added to 100 ml of a 1:1 mixture of acetone and butanol. This is the indicator used for detection of glycine production.

Just before use, approximately 0.025 ml (1 drop) of each sterile medium was added to sterile microtubes (disposable borosilicate glass, 6 by 50 mm; Kimble Div., Owens-Illinois, Inc.). The tubes were placed in microtiter V plates (Dynatech Laboratories, Inc.), and the media were allowed to warm to room temperature (22°C) before inoculation.

V agar. A total of 117 isolates confirmed as *G. vaginalis* were subcultured on V agar to evaluate the usefulness of this medium as described by Greenwood and Pickett (9).

Method of inoculation. With a 3-mm diameter loop, one third of a loopful of pure growth of each isolate grown for 18 to 24 h on Difco Gonococcus base plus 10% sheep blood agar or Columbia blood agar was inoculated into each of the three RM-SHR media. Particular attention was paid to emulsifying clumps of bacteria in the microtubes. Dispersal of organisms was accomplished by the mixing action of the loop. Without adequate emulsification and mixing, the reaction time was prolonged. After inoculation, the medium in the raffinose tubes was overlaid with 2 drops of sterile mineral oil. The oil overlay speeded up the fermentation reaction. Each of the isolates was also inoculated into CTA-based carbohydrates.

The inoculated tubes were then placed in microtiter plates and incubated at 36°C in a shallow water bath. The tests were read after 1 h of incubation. A positive starch or raffinose fermentation reaction was indicated by a yellow color, and a negative reaction was indicated by a red color.

In the hippurate test, 1 drop of ninhydrin solution was added to each tube containing hippurate medium. These tubes were reincubated at 36°C for 15 min and then observed. A positive hippurate test was indicated by a purple color after the 15-min reincubation period. A negative reaction was colorless. The maximum reincubation time of 15 min must be strictly adhered to, as prolonged incubation may result in false-positives.

TABLE 1. Biochemical reactions of 423 previously identified *G. vaginalis* and *G. vaginalis*-like isolates with CTA- and rapid micro-test-based substrates

Organism (no. of isolates)	No. (%) positive with following test and indicated substrate:						
	Rapid micro			CTA			
	Starch	Hippurate	Raffinose	Dextrose	Maltose	Lactose	Starch
<i>G. vaginalis</i> (396)	396 (100)	390 (98.5)	0 (0)	351 (88.6)	396 (100)	12 (3)	396 (100)
<i>G. vaginalis</i> -like (27)	4 (14.8)	6 (22.2) ^a	6 (22.2)	22 (81.5)	22 (81.5)	27 (100)	4 (14.8)

^a All hippurate-positive *G. vaginalis*-like strains were starch negative.

RESULTS

Of the 396 isolates previously identified as *G. vaginalis*, 390 fermented starch and hydrolyzed hippurate but did not ferment raffinose (Table 1). Six of these isolates (1.5%) fermented starch but failed to ferment raffinose or hydrolyze hippurate.

Of the 27 *G. vaginalis*-like isolates, 15 were starch, hippurate, and raffinose negative. Of the remaining 12, 6 were starch and raffinose negative but hippurate positive, 4 were starch and raffinose positive but hippurate negative, and 2 were positive only for raffinose.

CTA carbohydrate test. Of the 396 previously identified *G. vaginalis* organisms, 396 fermented starch and maltose, 351 fermented dextrose, and 12 fermented lactose (Table 1).

Of the 27 *G. vaginalis*-like organisms, all fermented lactose, 22 fermented dextrose and maltose, and 4 fermented starch.

V agar. Of 117 confirmed *G. vaginalis* isolates, 9 did not show beta-hemolysis when grown on V agar.

DISCUSSION

Various test schemes have been proposed for the identification of *G. vaginalis*; however, previously published work is inconsistent in terms of the number and selection of biochemical tests to be used for the identification of this organism. Although some studies used a larger number of tests for the identification of this species, the number of criteria necessary for differentiation and identification of *G. vaginalis* can be limited to a few (5).

The choice of biochemical tests used in the RM-SHR method appears to be valid in light of the results. For 98.5% of *G. vaginalis* isolates, the expected result of starch and hippurate positive and raffinose negative confirmed their identity. Six *G. vaginalis* isolates failed to hydrolyze hippurate, a result which is in agreement with the observations of Greenwood and Pickett (10), who earlier reported that a small percentage of *G. vaginalis* isolates do not hydrolyze hippurate. The same organisms, when tested by the CTA carbohydrate test method, gave the expected result of starch, dextrose, and maltose positive

and lactose negative for only 88.6% of the organisms, and a larger number of subsequent tests were required to properly characterize isolates which did not fit the expected biochemical profile.

As noted by previous workers (9), we also found that production of beta-hemolysis on V agar was a variable characteristic of *G. vaginalis*. Only 92.4% of the strains tested produced beta-hemolysis.

The 27 *G. vaginalis*-like organisms, when tested by the RM-SHR method, gave biochemical profiles for these three substrates which were different from the reactions expected for *G. vaginalis*. It is noteworthy that the six cultures in this group which hydrolyzed hippurate failed to ferment starch, and therefore, in contrast to the recommendation of others (10), we believe that the fermentation of starch is a useful identifying characteristic for *G. vaginalis*. The results obtained with the RM-SHR method underscore Smith's observation (21) of the value of including raffinose in a *G. vaginalis* identification scheme, since a positive reaction indicates a *G. vaginalis*-like organism.

Our proposed identification criteria, incorporating the RM-SHR test, should also prove beneficial in providing a means of distinguishing *G. vaginalis* from other organisms which are often isolated from the vaginal tract, such as *Lactobacillus* and *Bifidobacterium* species and diphtheroids (1, 5). Some of these organisms may, on the basis of initial tests and gram-variable staining characteristics, be presumptively identified as *G. vaginalis*. The criteria we suggest will provide the proper identification in such cases, since *Lactobacilli* rarely ferment starch (2), whereas *G. vaginalis* does. Aero-tolerant strains of *Bifidobacterium* do not hydrolyze hippurate, in contrast to *G. vaginalis*, which does in the majority of cases. Diphtheroids are catalase positive and usually starch negative. In addition, diphtheroids and *Lactobacilli* grow well on sheep blood agar medium (Trypticase soy agar (BBL) plus 5% sheep blood) in comparison with *G. vaginalis*, which grows poorly on blood agar.

In addition to establishing identification criteria, our study has demonstrated the advantage of the micro-technique in that reagents are easily

and economically prepared, produce clear-cut, unequivocal readings, and provide results within 1 h after inoculation.

We have found the use of the Gram reaction and oxidase and catalase tests, followed by the RM-SHR tests, to be a practical identification procedure in our laboratory and conclude that the RM-SHR method is a valuable diagnostic tool for clinical microbiology laboratories for the rapid identification of *G. vaginalis*.

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LITERATURE CITED

- Bailey, R. K., J. T. Voss, and R. F. Smith. 1979. Factors affecting isolation and identification of *Haemophilus vaginalis* (*Corynebacterium vaginale*). *J. Clin. Microbiol.* **9**:65-71.
- Dunkelberg, W. E. 1978. Isolation and identification of *Corynebacterium vaginale*. *J. Am. Med. Technol.* **40**:9-11.
- Dunkelberg, W. G., Jr., R. Shaggs, and D. S. Kellogg, Jr. 1970. Method for isolation and identification of *Corynebacterium vaginale* (*Haemophilus vaginalis*). *Appl. Microbiol.* **19**:47-52.
- Gardner, H. L. 1980. *H. vaginalis* vaginitis after twenty-five years. *Am. J. Obstet. Gynecol.* **137**:385-391.
- Gardner, H. L., and C. D. Dukes. 1955. *Haemophilus vaginalis* vaginitis. A newly defined specific infection previously classified "nonspecific." *Am. J. Obstet. Gynecol.* **69**:962-976.
- Gardner, H. L., and C. D. Dukes. 1959. *Haemophilus vaginalis* vaginitis. *Ann. N.Y. Acad. Sci.* **83**:280-283.
- Gordon, A. M., H. E. Hughes, and G. T. D. Barr. 1966. Bacterial flora in abnormalities of the female genital tract. *J. Clin. Pathol.* **19**:425-432.
- Greenwood, J. R. 1981. *Gardnerella vaginalis* (*Haemophilus vaginalis*, *Corynebacterium vaginale*), p. 23-25. In D. J. Blazevic, L. R. McCarthy, and J. A. Morello (ed.), *Clinical microbiology newsletter*, vol. 4. G. K. Hall & Co., Boston.
- Greenwood, J. R., and M. J. Pickett. 1979. Salient features of *Haemophilus vaginalis*. *J. Clin. Microbiol.* **9**:200-204.
- Greenwood, J. R., and M. J. Pickett. 1980. Transfer of *Haemophilus vaginalis* Gardner and Dukes to a new genus, *Gardnerella*: *G. vaginalis* (Gardner and Dukes) comb. nov. *Int. J. Syst. Bacteriol.* **30**:170-178.
- Greenwood, J. R., M. J. Pickett, W. J. Martin, and E. G. Macle. 1977. *Haemophilus vaginalis* (*Corynebacterium vaginale*). Method for isolation and rapid biochemical identification. *Health Lab. Sci.* **14**:102-106.
- Heltali, A., and P. Taleghany. 1959. Non-specific vaginal infections. *Am. J. Obstet. Gynecol.* **77**:144-148.
- Josey, W. E., and D. W. Lambe, Jr. 1976. Epidemiologic characteristics of women infected with *Corynebacterium vaginale* (*Haemophilus vaginalis*). *J. Am. Vener. Dis. Assoc.* **3**:9-13.
- Lapage, S. P. 1974. *Haemophilus vaginalis*, p. 368-470. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
- Lewis, J. F., S. M. O'Brien, U. M. Ural, and T. Burke. 1971. *Corynebacterium vaginale* vaginitis in pregnant women. *Am. J. Clin. Pathol.* **56**:580-583.
- McHardy, Elizabeth. 1979. The isolation of *Haemophilus vaginalis* (*Corynebacterium vaginale*) in the laboratory. *Can. J. Med. Technol.* **41**:77-83.
- Pheifer, T. A., P. S. Forsyth, M. A. Durfee, H. M. Pollock, and K. H. Holmes. 1978. Non-specific vaginitis. Role of *Haemophilus vaginalis* and treatment with metronidazole. *N. Engl. J. Med.* **298**:1429-1434.
- Plot, P., E. Van Dyck, M. Goodfellow, and S. Falkow. 1980. A taxonomic study of *Gardnerella vaginalis* (*Haemophilus vaginalis*) Gardner and Dukes 1955. *J. Gen. Microbiol.* **119**:373-396.
- Platt, M. S. 1971. Neonatal *Haemophilus vaginalis* (*Corynebacterium vaginale*) (sic) infection. *Clin. Pediatr.* **10**:513-516.
- Shtibel, R., and S. Toma. 1978. *Neisseria gonorrhoeae*: evaluation of some methods used for carbohydrate utilization. *Can. J. Microbiol.* **24**:177-181.
- Smith, R. F. 1979. Comparison of two media for isolation of *Haemophilus vaginalis*. *J. Clin. Microbiol.* **9**:729-730.
- Spiegel, C. A., R. Amsel, D. Eschenbach, F. Schoenknecht, and K. K. Holmes. 1980. Anaerobic bacteria in non-specific vaginitis. *New Engl. J. Med.* **303**:601-607.
- Yong, D. C. T., and A. Prytula. 1978. Rapid micro-carbohydrate test for confirmation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **8**:643-647.
- Zinnemann, K., and G. C. Turner. 1963. The taxonomic position of *Haemophilus vaginalis* (*Corynebacterium vaginale*). *J. Pathol. Bacteriol.* **85**:213-219.