

Laboratory diagnosis of *Gardnerella vaginalis* vaginosis

*R Nneka Okwoli, J. I. Brian-D Adinma and C. N. Nnaeze

*Department of Obstetrics and Gynaecology,

Department of Medical Microbiology,

Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria.

Summary

An evaluation of various laboratory detection methods and characteristics of *Gardnerella vaginalis* was made using high vaginal swab samples of 470 out patient clinic - attending women. Gram stain for 'clue cells' showed positive results in 118 (25.1%) cases; culture, in 100 cases, (21.3%) and Amine Odour (21.3%), in 26 cases 5.5%. Majority, 71 cases, of the culture-positive results were associated with a pH value of 6 to 7. *Gardnerella vaginalis* grew predominantly in enriched culture media: Modified peptone-starch dextrose blood agar used for primary culture of organism, and also proteose peptone broth + cooked meat; Brain-heart infusion broth + 5% human serum; Brain heart infusion starch agar + 5% blood, and chocolate agar. *Gardnerella vaginalis* culture-positive samples also exhibited positive biochemical reactions with the hydrolysis of starch sensitivity to Bacitracin and 50µg metronidazole, and haemolysis on human blood agar. Carbohydrate fermentation test was positive for all culture-positive cases, 100% for starch and maltose only, and negative for all the cases, 0% for Mannitol and glycerol.

Key words: *Gardnerella vaginalis*, Vaginal swab, Laboratory detection.

Résumé

Une évaluation des méthodes diverses de la découverte laboratoire pour, et les traits caractéristiques de Vaginalis Gardnerella ont été fait tout en utilisant des échantillons élevés des temps vaginaux de 470 femmes nigérianes qui viennent consulter à la clinique. Le gramstain pour cellule indice avait indiqué un résultat positif dans 118 cas; culture en 100 cas, et l'Odeur Amine dans 26 cas. La majorité 71 cas, des résultat positif-culture avaient des rapports avec une PH valeur de 6 à Viginalis Gardnerella qui est d'une manière prédominante a rehaussé le bouillon de culture; le sang agar de la peptone - amidon dextrose modifiée utilisé pour la culture primaire de l'organisme, et aussi le bouillon proteose-peptone + la viande cuite; l'infusion brouillon Brule-Vaginalis Gardnerella échantillons positifs-culture et aussi la présentation positive des réactions biochimique avec l'hydrolyse d'amidon très sensible à Bacitracine et 50 ng metronidazole; et hémolyse sur l'agar du sang humain.

La réaction de la fermentation d'hydrate de carbone était positif pour tous les case positifs-culture; 100% pour l'amidon et maltose seulement, et négatif pour tous les cas, 0% pour Mannitol et glycérol.

Le pluce relatif de ces traits diverses de la diagnostique laboratoire de Vaginalis Gardnerella et la critique de cette bibliographie sont ici l'objet de cette étude.

Introduction

Bacterial vaginosis (BV) is a relatively new sexually transmitted disease, the most important causative organism of which is *Gardnerella vaginalis* either alone or in combination with other organisms especially *Mycoplasma hominis*, *Mobiluncus* species and some obligate anaerobes.¹

G. vaginalis is important not just for its role in bacterial vaginosis but also for its involvement in several complications

affecting organs both in the pregnancy and non-pregnancy states, and even in males. It has been encountered in preterm labour, premature rupture of membranes and chorioamnionitis^{2,3} in neonatal meningitis⁴, following hysterectomy⁵ and also following prostatectomy.

Bacterial vaginosis is characterised clinically by the presence of adherent-grey, homogenous, offensive vaginal discharge; pH greater than 4.5, detection 'clue cells' in gram-stained smear, and the presence of amine odour on addition of potassium hydroxide^{7,8}.

In a previous report, we compared two laboratory methods of detection of *Gardnerella vaginalis*. The gram-stain for 'clue cells', and the GV culture methods. The present study in addition evaluates other laboratory diagnostic parameters for *G. vaginalis*, viz, pH, Amine odour test, growth in different culture media, biochemical characteristics, and carbohydrate fermentation reactions of *Gardnerella vaginalis* isolates.

Materials and methods

The study was conducted among 470 female patients attending the general out-patient, gynaecological and antenatal clinics of Nnamdi Azikiwe University Teaching Hospital (NAUTH) and Summit Specialist Hospital (a private medical centre), both in Nnewi, Anambra State of South-Eastern Nigeria, over the 15-month period, February 1994 to April 1995. The 470 cases consist of 253 consecutive ante-natal clinic patients with or without a complaint of vaginal discharge, and 147 general out-patient and gynaecological clinic patients complaining of vaginal discharge. Two high vaginal swabs (HVS) were collected for this study from the lateral and posterior vaginal fornices of the subjects. The following investigations were conducted.

Microscopy: Gram-stain and wet preparations of the smears were made to search for evidence of epithelial 'Clue cells'.

Amine odour test: A drop of 10% KOH was added to some vaginal discharge put in a clean slide. The slide was brought close to the nose and sniffed for the perception of fishy odour which was noted and recorded as positive amine odour test.

Inoculation of media and incubation: inoculation of the vaginal swab was done using standard plating method described by Cruikshank et al¹⁰. Primary culture was carried out on peptone-starch-dextrose blood agar made selective by the addition of 4mg Gentamycin; 15mg/L Nalidixic acid and 12.5iu/L Nystatin. The medium was incubated in candle jar which provided 5-10% carbon dioxide tension. An elevated humidity was provided by putting soaked filter paper or cotton wool inside the candle jar. Incubation was at 37°C. The primary plate was examined every 24 hours for 96 hours.

Gardnerella vaginalis appear as tiny greyish smooth, roundish colonies with zones of B-hemolysis after 48 hours on which Gram-stain showed either Gram-negative or variable cocco-bacilli. pH: pH was conducted on *G. vaginalis* culture isolate using universal pH indicator with colour code ranging from I to II. The isolated *Gardnerella vaginalis* was also cultured unto selected liquid and solid media, namely. Peptone-starch-dextrose broth; peptone-starch-dextrose agar; peptone-starch-dextrose sheep blood agar; chocolate agar; proteose peptone broth + cooked meat; proteose peptone broth + 5% human serum, Brain-heart infusion starch agar; brain-

*Correspondence

heart infusion starch agar + 5% blood; proteose-peptone agar and 3% sodium chloride agar.

Biochemical tests: Some biochemical tests were also performed on Gardnerella vaginalis culture isolates, viz:

- a. **Catalase test:** A drop of 3% hydrogen peroxide was added to G. Vaginalis isolate following 48 hours growth on chocolate agar. The presence of gas bubbles indicated catalase production.
- b. **Oxidase test^H:** A filter paper impregnated with 2 to 3 drops of 1% tetramethyl-phenylene-diamine dehydrochloride (oxidase re-agent) was placed in a petridish and a loopful of 48 hour growth of *G. Vaginalis* was smeared across the impregnated paper. A positive reaction was shown by the development of dark-purple colour within 10 seconds.
- c. **Indole test:** Kovac's colour reagent was added to 1 ml of 48 hour G vaginalis culture isolate. The presence of a red colour on the reagent layer on standing for 1 minute indicated indole production.
- d. **Citrate utilisation test:** A streak of G. Vaginalis was inoculated unto the surface of simmon's citrate agar slope and incubated aerobically at 37°C and examined daily for 7 days. A colour change from green to blue indicated positive reaction.
- e. **Urease test:** G. Vaginalis culture isolate was inoculated unto christensen's¹² agar slope and incubated aerobically at 37°C and examined daily for 5 days. The presence of a red colour indicated positive reaction.
- f. **Hydrolysis of Tween 80:** Heavy inoculation of G. vaginalis culture isolate unto Tween 80 agar plate was incubated in candle jar for 48 hours. Opaque haloes appearing under the colonies indicated positive result.
- g. **Hydrolysis of starch:** Gardnerella vaginalis culture was inoculated unto starch medium and incubated at 37°C. After 3 days the plate was flooded with Lugol's iodine solution. The presence of clear colourless zone indicate starch hydrolysis.
- h. **Hydrolysis of Gelatin:** Gelatin agar was inoculated with G. vaginalis culture isolate and incubated at 37°C in carbon dioxide for 3 days. The plates were then flooded with mercuric chloride solution. The presence of opacity in the medium with clear zones around colonies indicate gelatin liquifaction.
- i. **Antibiotic diagnostic test:** Using a sterile cotton wool swab, a 48 hour broth culture of the organism was inoculated onto chocolate agar plates with Bacitracin. 5 mg and 50mg metrodanizole discs. This was incubated in carbon dioxide at 37°C for 48 hours, after which sensitive discs are shown as zones of inhibition while resistant ones show no zones of inhibition.
- j. **Haemolysis test:** This was done by inoculating the G. vaginalis culture isolate unto peptone-starch dextrose human blood agar, and peptone-starch dextrose sheep blood agar. The haemolytic ability to these two blood-types was noted.
- k. **Acid production from carbohydrates:** The method of greenwood and Pickett¹³ was used to prepare the medium for carbohydrate fermentation. Culture bottles containing the medium were heavily inoculated by stabbing with 48 hour cultures of G vaginalis grown on peptone-starch-dextrose blood agar. The bottles were incubated aerobically for 5 days. Acid production was indicated by a yellow colour. Sugars used for this test include: Arabinose, Dextrose, Galactose, Glycerol, Lactose, Maltose, Mannitol, Starch and Sucrose.

Result

The distribution of the result of Gram-stain (for 'Clue cells'); culture, and amine odour is shown in Fig 1. Gram-stain yielded positive result in 118 cases, out of which culture positivity occurred in 100 cases, and in which yet amine odour positivity occurred in 26 cases.

The distribution by pH for G vaginalis culture-positive result is shown in Table 1. All the culture positive results exhibited an alkaline pH reaction. The dominant p^H was 6 (44 cases).

The culture and growth of G. Vaginalis in different growth media as shown in Table 2 indicates positive result in the following media: proteose-peptone-broth + 5%

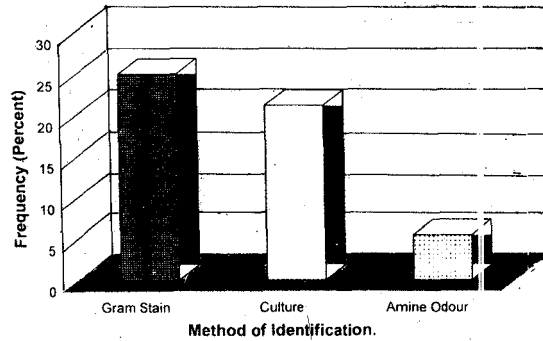


Fig. 1 Distribution by positive Gram-stain and Amine odour

Table 1 Distribution by pH for gardnerella vaginalis culture-positive result

pH	No. culture-positive	Percent	n=100
5	21	21	
6	44	44	
7	27	27	
8	8	8	
Total	100	100	

Table 2 Culture and growth of gardnerella vaginalis to different growth media

Medium	Growth Result
Brain-heart infusion broth	-ve
Nutrient broth	-ve
Proteose-peptone broth	-ve
Proteose peptone broth + 5% human serum	+ve
Proteose peptone broth + cooked meat	+ve
Brain heart infusion broth + 5% human serum	+ve
Nutrient agar	-ve
Mackonkey agar	-ve
Brain heart infusion agar	-ve
Brain heart infusion starch agar	-ve
Brain heart infusion starch agar + 5% blood	+ve
Proteose peptone agar	-ve
3% sodium chloride agar	-ve
Chocolate agar	+ve

Key: +ve - Positive -ve - Negative

Table 3 Biochemical tests for gardnerella vaginalis culture isolates

Test	Result	n = 10
Catalase	-ve	
Oxidase	-ve	
Urease	-ve	
Indole	-ve	

Key: -ve - Negative

human serum; brain-heart infusion starch agar + 5% blood, and chocolate agar. All the other growth media yielded negative result to G vaginalis culture.

Table 3 shows the Biochemical Test of the culture-positive samples. All the tests - catalase, oxidase, urease and indole show negative reaction. Hydrolysis, sensitivity and haemolysis tests for Gardnerella vaginalis culture isolates is shown in table 4. Positive reactions were exhibited, in the hydrolysis of starch; sensitivity to Bacitracin and 50µg metronidazole and haemolysis on human blood

agar. Negative reactions occurred in all the other biochemical tests. Table 5 shows the distribution by carbohydrate fermentation tests of *Gardnerella vaginalis* culture isolates. Fermentation test was positive for starch and maltose in all the cases 100(100%). This was followed by dextrose, 89(89%) cases, Lactose, 72 (72%) cases, Galactose, 70 (70%) cases; sucrose, 34 (34%) cases; and Arabinose, 28 (28%) cases.

Table 4 Hydrolysis, sensitivity and haemolysis tests, for *Gardnerella vaginalis* culture isolates

Test	Result	n = 100
Hydrolysis of:		
Tween 80	-ve	
Gelatin	-ve	
Starch	+ve	
Sensitivity to:		
Bacitracin	+ve	
Metronidazole (5µg)	-ve	
Metronidazole (50µg)	+ve	
Haemolysis on:		
Sheep blood agar	-ve	
Human blood agar	+ve	

Key: +ve - Positive, -ve - Negative

Fermentation test was not positive at all in any case (0%) for mannitol glycerol.

Table 5 Distribution by carbohydrate fermentation tests on *Gardnerella vaginalis* culture isolates

Carbohydrate	No. Positive	Percent	n=100
Arabinose	28	28.0	
Sucrose	34	34.0	
Dextrose	89	89.0	
Mannitol	0	0.0	
Galactose	70	70.0	
Maltose	100	100.0	
Lactose	72	72.0	
Starch	100	100.0	
Glycerol	0	0.0	

Discussion

The clue cell phenomenon, one of the identification features of bacterial vaginosis is due to attachment of adherent strains of *Gardnerella vaginalis* to epithelial cells¹⁴ Gram-staining for 'clue cells' has been shown to be a rapid, acceptable routine screening method for the identification of *G. vaginalis*. It is believed to be specific for *G. Vaginalis* vaginosis¹⁵⁻¹⁷. It is cheap and correlates well with culture results and has therefore been unequivocally recommended for use in developing countries where culture facilities are lacking 'Clue cell' was positive in 118 cases in the study. Gram-staining therefore exhibited better detection properly for *Gardnerella vaginalis* than culture which identified 100 cases and amine odour test which identified only 26 cases.

Amine odour test carried out in the study showed poor correlation with the incidence of *G. Vaginalis* isolated from culture, being positive only in 26 cases. Similar observation was made by Saini et al who therefore emphasized the necessity for culture in the definitive diagnosis of *G. vaginalis* vaginosis. In addition, Jones et al¹⁹ had stressed on the non-specificity of amine odour test which may also be positive in *Trichomonas vaginalis* infection. In contradiction to the above, Abudu et al²⁰ found Amine odour test to be positive in 77.3% of their cases and recommended it as a mandatory test in a set of tests for the screening of *Gardnerella vaginalis*. Chen et al²¹, had identified 7 amines from the vaginal washings of patients with bacterial vaginosis among which putrescine and cadaverine are in highest concentration. The amines are derived from the decarboxylation activity of anaerobes on amino acids and pyruvic acid produced by *Gardnerella vaginalis*. They enhance the growth of the anaerobes, increase the concentration of amines in vaginal secretions and actually contribute to the patho-

genesis of bacteria vaginosis and elevation of the p^H of vaginal discharge. They may also be partly responsible for the fishy odour that characterise the vaginal discharge of these patients²²

A p^H greater than 4.5 is one of the universally accepted criteria for the diagnosis of bacterial vaginosis⁸. Majority of the *G. vaginalis* isolates, 71(71%) in this study was associated with p^H between 6 and 7.

Gardnerella vaginalis is a highly fastidious organism and requires an enriched media for culture. Primary culture of the organism was performed in this study using a modification of peptone-tarch-dextrose agar²³ made selective by the incorporation of Gentamycin, Nalidixic acid and Nystatin. Blood was added to the medium to demonstrate haemolysis and also to enhance the growth of the organism. Many workers have used Columbia blood agar with Nalidixic acid and colistin sulphate. That an enrichment factor is necessary for enhanced growth of this organism is demonstrated in this study from the result of *G. vaginalis* growth in different media, for instance, no growth was observed in ordinary brain-heart infusion broth while growth occurred when human serum was added to the broth. Similar result was observed in the solid media where growth was observed in brainheart infusion agar only when blood was incorporated. In media lacking blood, such as the media for carbohydrate fermentation, citrate and gelatin tests, heavy inoculum was used and incubation had to be over a longer period (3 or more days), for any significant growth to be observed.

Gram-staining of *G. vaginalis* culture isolates show Gram-variable coccobacilli which appear singly, in pairs, or in pallicade arrangement. The Gram-variable nature of *Gardnerella vaginalis* was confirmed from recent report by Catlin²⁴ following electron microscopy and chemical analysis of the organism. Prior to this time, the taxonomic position of *Gardnerella vaginalis* was under a lot of controversy. Earlier workers^{7,16} had labelled it as Gram-negative while others²⁵ had regarded it as Gram-positive organism.

Biochemical identification tests and carbohydrate fermentation tests performed in this study were mostly in accordance with those performed by Greenwood and Pickett¹³ and Taylor and Philips²⁶. Although variable results were observed with some of the sugars used, maltose and starch remained consistently 100% positive to fermentation while glycerol and mannitol remained consistently negative (0%). Sensitivity to Bacitracin¹³ and resistance to lower concentration of metronidazole²⁶ are used as identification tests. In this study *G. Vaginalis* isolates were resistant to 5ug metronidazole and sensitive to bacitracin and 50ug metronidazole. This agrees with the findings of Pandit et al²⁷ who reported that none of their isolated strains of *G. vaginalis* was sensitive to 5ug metronidazole while 93% were sensitive to 50ug metronidazole discs. Well and Goei²⁸ recommended the use of a combination of tests which include hydrolysis in sheep blood agar; hippurate hydrolysis; inhibition by bacitracin and *Streptococcus sanguis*; and susceptibility of metronidazole at 50ug and resistance to 1.5ug sulphonamide for identification of *G. vaginalis*. Young and Thompson²⁹ utilised the fact that most isolates of *G. vaginalis* hydrolyse hippurate and ferment starch but not raffinose to develop a Rapid microbiobiochemical method for the identification of *G. vaginalis* called a Rapid micro-starch-hippurate-raffinose (RM-SHR) method. The medium, now commercially available is now called a Rapid identification method (RIM).

Recently, Sheiness et al³⁰ demonstrated a new approach to the diagnosis of bacterial vaginosis based on measuring the concentration of *G. Vaginalis* in vaginal fluids using DNA probes will not cross hybridise with DNA of a number of non-*Gardnerella* organisms commonly found in the vagina thereby making this method a useful tool for the direct identification of *G. Vaginalis* from mixed clinical specimens. Indirect immuno-fluorescent technique, using fluorescein-labelled *G. vaginalis* polyclonal antibody detected *G.*

vaginalis in vaginal smears in a higher percentage than its culture³². Cytological studies have also been used to identify *G. Vaginalis*^{33,34}. In conclusion, this study has recognised the important place of *Gardnerella vaginalis* as a cause of infection in humans, and highlighted different characteristics of the organism and possible laboratory diagnostic methods for its identification. It also reviews the place of these identification methods as well as highlight other available modern; simple, sophisticated; or rapid detection methods for *Gardnerella vaginalis*, and for a more accurate diagnosis of bacterial vaginosis.

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