Detection of *Gardnerella vaginalis* in Vaginal Specimens by Direct Immunofluorescence

WILLY HANSEN,†* BERNARD VRAY,2 KARIN MILLER,† FRANCOISE CROKAERT,† AND EUGENE YOURASSOWSKY†

Department of Microbiology, Brugmann University Hospital, 1020 Brussels,† and Laboratory of Immunology, Faculty of Medicine, Free University of Brussels, 1000 Brussels,2 Belgium

Received 12 January 1987/Accepted 1 July 1987

*Gardnerella vaginalis* is the predominant organism isolated from patients presenting with nonspecific vaginitis (2, 4, 7, 10-13, 17, 21). In addition, some workers have suggested that the nonspecific vaginitis is in fact a more complex process involving an unknown relationship between *G. vaginalis* and other anaerobic vaginal bacteria (21, 22). The term bacterial vaginosis has been proposed (8).

Isolation of *G. vaginalis* from clinical material requires complex media, which are difficult to prepare. Owing to the drawbacks that affect most of the methods described so far, we developed a direct fluorescent-antibody technique which allows the detection of the organism in clinical samples and the rapid identification of suspected colonies in cultures.

**MATERIALS AND METHODS**

**Preparation of the conjugate.** (i) Preparation of the immunizing material. *G. vaginalis* strains were obtained from the American Type Culture Collection (strains ATCC 14018 and ATCC 14019). These strains were harvested from "V" agar (23) and suspended in a saline solution. The cells were centrifuged (at 1,000 × g) and resuspended in saline solution to yield approximately 2 × 10⁹ bacterial cells per ml on the basis of the cell concentration of a no. 7 McFarland standard. No adjuvant was added.

(ii) Immunization protocol. Two young adult male rabbits (Flemish giants) were used for immunization, each with one reference strain. Preimmunization serum was obtained from each animal and preserved as a control to assess the specificity of the staining reaction.

The protocol of immunization was as follows: day 1, 1.5-ml intramuscular injection in the shoulder; day 3, 0.5-ml intravenous injection in the major vein of the external ear; day 4, 1-ml intramuscular injection; day 5, 1.5-ml intravenous injection; day 7, 1.5-ml intramuscular injection; day 10, 1.5-ml intravenous injection; and day 13, 1.5-ml intravenous injection. Blood was withdrawn 10 days after the last booster, and afterwards the animals were boostered once a month and bled twice.

Antibody titers, determined by indirect immunofluorescence by using goat anti-rabbit serum purchased from Burroughs Wellcome Co., ranged from 1/800 to 1/1,200.

(iii) Adsorption of cross-reacting agglutinins. To avoid cross-reactions, the antisera were adsorbed with the following clinical isolates, obtained from vaginal specimens: *Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus jensenii, Corynebacterium pseudodiphtheriticum, Corynebacterium xerosis,* and *Corynebacterium group JK,* bacteria usually recovered from the vaginal cavity and exhibiting mostly similar morphologies. These bacteria were grown separately for 24 h on heart infusion agar and washed with saline three times. The pellets from the last centrifugation of all the bacteria were then suspended together in one suspension of 2 × 10⁹ CFU/ml in phosphate-buffered saline. The immune serum and the adsorption mixture were mixed in equal volumes. The mixture was stirred for 4 h at 37°C, for 2 h at room temperature, and finally overnight at 4°C. The nonspecific antibody complexes were eliminated by centrifugation (1,000 × g, 4°C, 20 min). None of the adsorbing strains was detected by indirect immunofluorescence with the adsorbed serum.

(iv) Preparation and testing of fluorescent conjugates. Immunoglobulin fractions were obtained by two successive precipitations with ammonium sulfate at 33% final concentration. The immunoglobulins were then labeled with fluorescein isothiocyanate (obtained from BDH). The amount of fluorescein isothiocyanate used for labeling was 25 μg/mg of proteins (6, 15).

To test the conjugates, 5 homologous bacterial strains (including the 2 initial vaccine strains) and 23 verified heterologous bacterial strains obtained from vaginal specimens (Table 1) were suspended in saline to a turbidity equivalent to that of a McFarland 0.5 standard. One-drop samples of the suspensions were placed onto microscope slides and air dried. The bacteria were covered with 1 drop of the diluted conjugate (dilutions are given in Table 1), incubated at 37°C in a moist chamber for 30 min, and washed with phosphate-buffered saline for 10 min. The preparations were mounted in glycerol phosphate-buffered saline (9:1, vol/vol) and examined with a Leitz Dialux microscope equipped with a KP 500 blue filter. The light source was an HBO 50-W alternating current Osram lamp with mercury vapor. The specimens

* Corresponding author.
were read with a magnification of \( \times 1,000 \) (lens 100/1; 20-W Fluorezenz Leitz Wetzelr). The degree of bacterial immunofluorescence was graded from 0 to 4+ on the basis of the intensity of bacterial immunofluorescence, as follows: 1+, indistinctly fluorescent periphery and non- or weakly fluorescent center; 2+, slight fluorescence of the entire bacterial cell and weakly or discontinuous fluorescent periphery; 3+, center slightly fluorescent with a continuous distinctly fluorescent periphery; 4+, brilliant and continuous fluorescent periphery with non- or slightly fluorescent center. All the conjugates were stored at \(-20^\circ C\) in small vials. The titers of the fluorescent conjugates were defined as the lowest dilution giving a detectable immunofluorescence. The fluorescein-to-protein ratio was measured by the methods of Hebert et al. (6) and Hudson and Hay (9).

**Bacteriological study.** (i) Patients. A total of 263 vaginal swabs from patients attending the gynecological consultation unit of a general hospital were studied. Women who were pregnant or menstruating or who had received antimicrobial agents within the previous months were excluded. Of these women, 66 harbored an intrauterine device (IUD), among whom 32 were completely asymptomatic; the patients harboring IUDs were subjected to a thorough study. Clinical information was not available for the other patients.

(ii) Collection of specimens and bacteriological study. Vaginal specimens were collected in tandem by using two charcoal cotton swabs and Stuart’s transport medium; one swab was used for culture, and the other was used for immunofluorescence examination. The swabs for culture were plated on two specific media: modified starch differential medium (16) and human blood agar medium (“V” agar) (20); the plates were incubated for 48 h at 37°C in an anaerobe jar (GasPak; BBL) (14). After incubation, *G. vaginalis* appears as small, colorless, hemolytic colonies on human blood agar plates (5). On starch differential medium agar plates, the colonies become yellowish as a result of the production of acetic acid, which is the major end product of acid fermentation accumulated in anaerobic cultures (14). In this study, *G. vaginalis* was identified by the following characteristics: (i) its susceptibility to metronidazole at 50 \( \mu \)g per disk (Oxoid) (any zone, according to Piot et al. [19]), (ii) growth inhibition by sodium polyanetholsulfonate-impregnated disks (prepared by impregnation of disks with 20 \( \mu \)l of an aqueous 5% solution [L. G. Reimer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C29, p. 316]), and (iii) absence of catalase activity but clearly detectable hippurate hydrolysis (18). Nonbacteriological studies, such as pH, KOH “sniff test,” or chromatography, were not performed on the vaginal discharges in this study; however, a Gram stain was systematically performed in patients harboring IUDs to make evident the presence of clue cells (and the lack of polymorphonuclear leukocytes).

(iii) Application of conjugates to the detection of *G. vaginalis* on vaginal specimens. The vaginal specimens were smeared on slides, air-dried, and fixed with methanol at room temperature. Slides were incubated in a dark moist chamber for 30 min at 37°C with 5 \( \mu \)l of the conjugate. Then they were washed and soaked twice in phosphate-buffered saline for 10 min, rinsed again in distilled water, air dried, and mounted on a coverslip with glycerol (pH 9.0). Smears were examined with the help of a fluorescence microscope, and the degree of bacterial immunofluorescence was graded from 0 to 4+ on the basis of the intensity; a 3+ to 4+ degree of immunofluorescence was considered highly significant.

(iv) Identification of suspected colonies in cultures. A suspected colony was sampled with the end of a straight inoculating wire, and the portion was emulsified in a small drop of water on a microscope slide. After the slide had air dried, the immunofluorescence test was performed as explained above, and the degree of immunofluorescence was graded from 0 to 4+ on the basis of the intensity; a 3+ to 4+ degree of immunofluorescence was considered highly significant.

**RESULTS**

**Characteristics of the conjugates.** The individual antisera, obtained before each intravenous injection during the immunization period, were tested separately. The antibody titers varied from one rabbit to another during the immunization process. However, the immune response was enhanced after each booster before reaching a plateau. The immunoglobulin concentration increased during the immunization procedure from 5 to 18 mg/ml. Only sera of high titer from individual bleedings were conjugated. The dye/protein ratio of the conjugates was 2:1:1. Direct staining titers of the conjugate were positive as far as 1/128 after the adsorption procedure of the serum on mixed bacteria, which decreases by one-half the titer of the serum. No difference was observed between the conjugates obtained by immunization of rabbits with strain ATCC 14018 or ATCC 14019.

**Specificity and sensitivity of the conjugates.** The conjugates identified all 35 of the *G. vaginalis* strains, tested, with a high intensity of fluorescence. To obtain data on the specificity of
TABLE 2. Results of the detection of G. vaginalis in 263 clinical specimens by the immunofluorescence test and by culture technique

<table>
<thead>
<tr>
<th>IF result</th>
<th>No. (%) of cultures for G. vaginalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>33 (12.5) 22 (8.4)</td>
</tr>
<tr>
<td>Negative</td>
<td>0 (0.0) 208 (79.1)</td>
</tr>
</tbody>
</table>

* IF, immunofluorescence.

the conjugates, as well as information about possible cross-reactions, we screened smears of 23 pure cultures of bacteria isolated from clinical specimens with dilutions of the Gardnerella conjugate (Table 1). A cross-reaction was observed with Candida albicans. However, the morphological characteristics clearly ruled out any confusion between the identities of both microorganisms.

A total of 263 clinical specimens were examined in this study. Fluorescence for G. vaginalis was noted in 55 of them (20.9%), whereas a positive culture was noted in only 33 (12.5%) (Table 2). However, it must be pointed out that the fluorescence was of different degrees for the 22 patients with negative cultures. Among the 66 patients harboring IUDs, immunofluorescence was observed in 23 vaginal specimens (34.8%), whereas clue cells (together with lack of polymorphonuclear leukocytes) were observed in 19 of the corresponding Gram-stained smears. Clue cells were not observed among the 43 patients harboring IUDs who were negative for G. vaginalis immunofluorescence and culture.

In this IUD-harboring population, G. vaginalis-positive cultures were obtained from 20 of the 23 immunofluorescence-positive specimens. In the 197 remaining patients who did not have an IUD, immunofluorescence was observed in 32 vaginal swabs (16.2%). G. vaginalis-positive cultures were observed in 13 of the 32 immunofluorescence-positive specimens.

A high intensity of fluorescence (3+ to 4+) was observed when the immunofluorescence technique was used for the identification of suspected colonies in the 33 cultures which later turned out to be positive for G. vaginalis by identification methods using biochemical tests.

DISCUSSION

Rabbit immunization with G. vaginalis resulted in a specific immune serum allowing direct immunofluorescence detection of this organism in vaginal smears.

As expected, a high variability was observed in the titers of antibodies, and only the blood samples exhibiting high titers of antibodies were retained and stored. Furthermore, purification and labeling procedures induced a reduction in the titers of the immune serum. No differences were observed between conjugates from strain ATCC 14018 or ATCC 14019; use of the fluorescent conjugate at a high dilution is recommended to avoid cross-reactions, especially with C. albicans. The low intensity of fluorescence was not a major inconvenience since the confusion of C. albicans with G. vaginalis was avoided because of their very dissimilar morphologies. The prevalence of G. vaginalis in different studied populations is high. In 1970, Dunkelberg et al. (3) reported an incidence of 31% in 200 women attending a sexually transmitted diseases clinic, whereas the incidence determined by Stoerjelt et al. in 1983 (24) was higher: 46.9%. Cano et al. (1), who developed an indirect immunofluorescence-antibody test, observed an incidence of 24.2% in patients with nonspecific vaginitis, whereas G. vaginalis was detected in 29.8% of the vaginal smears from asymptomatic individuals. G. vaginalis was isolated from the urethras of men with an incidence of 10.5% (23). The direct immunofluorescence technique on vaginal specimens suggests in the present study that 21% of the patients harbored G. vaginalis. The organism was isolated from only 12.5% of the samples. Death of the organisms or inhibition by a polymicrobial saprophytic flora of rapid growth rate could not be ruled out in the cases of negative cultures. Pure cultures were subsequently obtained by classic and traditional subculture methods, allowing afterwards the identification by appropriate biochemical tests, but the risk of losing the strains during the subcultures is very important. In the meantime, a positive immunofluorescence test increased the probability of detecting G. vaginalis easily in cultures. The immunofluorescence technique for the identification of microorganisms, especially of G. vaginalis, was simple and rapid; the results can be obtained within 1 h. The use of this conjugate showed a high incidence (21%) of G. vaginalis in the total population that we studied. However, this frequency was higher among the IUD-harboring patients; 23 of 66 (34.8%) exhibited a positive immunofluorescence. In this particular group, G. vaginalis could not be isolated from three of the immunofluorescence-positive samples (two patients complained of discharge, whereas the third one was asymptomatic). However, all three of these Gram-stained smears showed evidence of bacterial vaginosis (presence of clue cells and abundant mixed aerobic and anaerobic flora, as well as presence of bacteria suggesting Mobiluncus spp., which were lost on culture).

Concerning the 18 additional immunofluorescence-positive-culture-negative smears, the relationship with the clinical picture was not available. It must also be pointed out that 22 samples showed fluorescence. The finding of immunofluorescent samples associated with negative cultures could be due to different factors: (i) lack of specific and sensitive selective media; (ii) death of the organism in transport; (iii) inhibition of G. vaginalis by rapidly growing bacteria or inability to distinguish the organism in a heavily mixed culture; and (iv) cross-reactivity occurring with other microorganisms, such as Mobiluncus spp. or other fastidious bacteria. On the other hand, antigenic differences between strains isolated from humans and reference strains could be responsible for the weak fluorescence reactivity, although this fact does not explain the negative cultures. The immunofluorescent conjugates recognized G. vaginalis in a mixed bacterial population directly on vaginal smears or on cultures; the identification by the immunofluorescence technique of suspected colonies which later turned out to be G. vaginalis always exhibited clear-cut positive reactions. It is a rapid diagnostic tool and draws attention to possibly positive cultures. In addition, this fluorescent conjugate can be easily used in epidemiological investigations. Finally, the relevance of the technique lies in the fact that G. vaginalis can be detected in specimens from patients treated with antibiotics and whose cultures remain negative.

ACKNOWLEDGMENTS

We thank Suzy Vekemans for technical assistance, Max Labbé for excellent advice, and Thierry Hubert for obtaining the specimens and the clinical information. The secretarial assistance of Yvonne Bawens and Lydia Fauconnier is gratefully acknowledged.
LITERATURE CITED


