Analysis of *Corynebacterium vaginale* by an Immunodiffusion Technique

MARY F. SMARON and JOHN L. VICE

Departments of Pathology and Microbiology, Loyola University Medical Center, Maywood, Illinois 60153

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An immunodiffusion technique was employed to study the antigenic relationship of *Corynebacterium vaginale* isolates, vaginal diphtheroids, and members of the genera *Corynebacterium* and *Lactobacillus*. Antisera were prepared against *C. vaginale* ATCC strain 14018 grown diphasically and on blood agar plates and were tested against extracts of organisms prepared by sonication. Ouchterlony analysis demonstrated that all of the isolates of *C. vaginale* examined possess a common antigenic determinant. No antigenic relationship was detected between *C. vaginale* and members of the genera *Corynebacterium* or *Lactobacillus*. This study also demonstrated that alterations in the cultural conditions can cause variations in the antigenic composition of *C. vaginale*.

Leopold (6) and Gardner and Dukes (5) isolated an unclassified organism from cases of vaginitis and urethritis and proposed that the organism was a member of the genus *Hemophilus* on the basis of its Gram-stain reaction, growth requirements, and microscopic and colony morphology. Subsequent morphological, biochemical, and serological studies failed to confirm the resemblance between this organism and members of the genus *Hemophilus* and, furthermore, led several investigators to designate the organism *Corynebacterium vaginale* (2, 8).

A major problem in evaluating the taxonomic position of *C. vaginale* and its role in disease is the difficulty encountered in the isolation and differentiation of *C. vaginale* from other diphtheroid-like organisms. Methods based on morphological and biochemical tests (3) have proven to be time consuming and subject to error in interpretation. However, we have found the indirect fluorescent-antibody technique to be a simple and rapid method for the presumptive identification of *C. vaginale* (7).

The present immunodiffusion study was undertaken to investigate the antigenic relationship of various strains of *C. vaginale* and possible related bacteria. Although fluorescent microscopy is a rapid technique, it lacks the resolving power of the immunodiffusion technique which has the ability to recognize numerous antigens and antibodies in mixtures and to establish the identity of antigens.

In the Ouchterlony analyses, sonicated cells were used as the source of antigen, thereby making available both extracellular and intracellular antigens to the system and increasing the chances of making fine distinctions.

Comparison of the antisera prepared against the blood and diphasically grown organisms revealed that the type of media employed does play a major role in the production of a specific antigen(s) by *C. vaginale*. The present study also demonstrated that all of the 14 *C. vaginale* isolates examined share a common antigen. No antigenic relationship could be demonstrated between *C. vaginale* and the vaginal diphtheroids or species of the genus *Corynebacterium* or *Lactobacillus* examined. This suggests that the Ouchterlony technique could be employed as a specific method for the presumptive identification of *C. vaginale*.

**MATERIALS AND METHODS**

**Organisms.** Six reference strains of *C. vaginale* were employed in this study. W. E. Dunkelberg supplied three strains (594 D, 6488 D, and T94), R. E. Weaver forwarded two strains (6488 W and 8226), and type strain 594 (ATCC no. 14018) was obtained from ATCC. P. Pease provided *C. cerucis* strain 13. Also obtained from the ATCC were *C. diphtheriae* strain 11913, *C. xerosis* strain 7711, and *Lactobacillus acidophilus* strain 4356. Nine clinical isolates, two obtained from W. E. Dunkelberg (V28 and V44) and seven isolated in our laboratory (144, 359, 1544, 1575, 1637, 6234, and 8315), that gave a positive indirect fluorescence with anti-*C. vaginale* antiserum (7) and morphologically and biochemically resembled *C. vaginale* were included in this study. Three vaginal diphtheroid-like organisms (8372, 417, 6659) that gave a negative fluorescence and biochemically did not resemble *C. vaginale* were also included.

**Media.** *C. vaginale* type strain 594 (ATCC no. 14018) was grown on blood agar plates containing 5%
sheep red blood cells (BBL). The plates were incubated at 37 °C under increased carbon dioxide tension in a candle jar for 48 to 72 h. C. vaginale isolates and the vaginal diphtheroid-like organisms were also grown diphasically. The diphasic medium employed, previously described (7), was composed of peptone-starch-dextrose agar in the solid phase overlayed with thioglycolate broth. Flasks were inoculated heavily with organisms taken from blood agar plates. The flasks were incubated at 37 °C for 48 to 72 h.

*L. acidophilus* was also grown diphasically. The diphasic medium was composed of Eugon broth and Eugonagar (BBL). Incubation time, amounts, and method of preparation were the same as described for *C. vaginale* diphasic medium (7).

*C. diphtheriae*, *C. xerosis*, and *C. cervicis* were grown on blood agar plates for 24 to 48 h at 37 °C.

**Antisera.** Antisera were prepared against *C. vaginale* ATCC no. 14018 strain grown on blood agar plates (anti-14018 Bld) and diphasically (anti-14018 Di). The method of antisera preparation was previously described (7).

**Ouchterlony studies.** Antigens were prepared by washing bacteria four times with normal saline (0.85% NaCl) followed by a final washing with distilled water. The cells were resuspended in 10 ml of distilled water and were then disrupted in an ultrasonic oscillator (Heat Systems, Ultrasonics Incorporated, Plainview, N.Y.) operating at 20 Kcycle/s to produce at least 50% cell disruption as revealed by phase-contrast microscopy. Each of the organisms to be tested was subjected to various sonication times, starting at 5 min and increasing at 5-min intervals to a maximum of 40 min. *C. vaginale* cells required 30 min of sonication; the other *Corynebacterium* required 5 to 10 min of sonication, and *L. acidophilus* required 15 min. Sonication was performed with cell suspensions immersed in baths of aceton and dry ice. The probe (0.5 inch [1.27 cm]) was cooled periodically. Sonically treated material was centrifuged for 15 min at 27,000 × g. Supernatants were lyophilized and reconstituted to a concentration of 1 mg (dry weight) per 0.1 ml of distilled water. A negative control was prepared by removing the broth from an uninoculated flask and treating it as described above.

The liquid phase from inoculated diphasic media was also utilized as a source of antigen for the Ouchterlony studies. The broth from a 48- to 72-h culture was dialyzed against repeated changes of water at 4 °C for 3 days. The dialysate was centrifuged at 27,000 × g for 20 min, and the clear supernatant was lyophilized and reconstituted with 5 ml of distilled water. Uninoculated broth processed in the same manner was used as a negative control.

Immunodiffusion plates were prepared by pouring 11 ml of agar onto 3.25- by 4-inch (8.26- by 10.16-cm) glass slides or 5 ml of agar onto 1- by 3-inch (2.54- by 7.62-cm) glass slides. The agar had the following composition (in grams per liter): ion agar, 10; sodium chloride, 8.5; sodium azide, 1; glycine 37.5. Wells were cut in the agar so that the antiseraum well measured 6 mm and the antigen well measured 3 mm. The peripheries of the wells were 5 mm apart. Reactions were allowed to proceed for up to 72 h at room temperature in a moist chamber. Slides were washed in saline for 24 h and then washed in distilled water for 24 h. After removal from the water, the slides were covered with filter paper and allowed to air-dry.

Slides were stained for 30 min by using napthalene black 12 B dye of the following composition: dye, 1 g; methanol, 500 ml; glacial acetic acid, 200 ml; and water, 500 ml. Slides were destained for 5 to 10 min in methanol-water-glacial acetic acid in a volume ratio of 7:3:1.

Preliminary experiments were performed to determine the optimum dilutions for immunodiffusion. Lyophilized supernatants from sonicated organisms were reconstituted with distilled water, and serial two-fold dilutions were tested against anti-*C. vaginale* antisera. In all cases, the most distinct precipitin bands were produced when extracts were utilized at a concentration of 1 mg (dry weight)/ml of distilled water.

**RESULTS**

**Effect of growth media on production of antigens.** To determine whether the type of growth media employed had an effect on the production of antigens by *C. vaginale*, extracts of sonicated cells of *C. vaginale* strain 14018 grown diphasically and on blood agar plates were reacted with antisera prepared against this organism grown on both types of media. As shown in Fig. 1A, extracts of diphasically grown cells (wells 2, 4, and 6) gave rise to two well-defined precipitin bands when reacted with antisera prepared against the organism grown on blood (anti-14018 Bld). In contrast, extracts of the blood-grown cells (wells 1, 3, and 5) gave rise to a single precipitin band that coalesced with the "inner" band produced by the diphasically grown cells (wells 2, 4, and 6). Antiserum prepared against the same strain of *C. vaginale* grown diphasically (anti-14018 Di) produced two precipitin bands with the extract of the blood-grown cells (Fig. 1B, wells 1, 3, and 5) and with the extract of diphasically grown cells (Fig. 1B, wells 2, 4, and 6). A ring of identity formed early with the inner precipitin bands and is visible in Fig. 1B. With increase in time the outer bands also coalesced indicating identity.

The broth extract (Fig. 2, wells 1 and 4) of *C. vaginale* strain 14018 also produced a precipitin band when reacted with anti-14018 Di. This band coalesced with bands produced by sonicated extracts of 14018 cells grown either on blood agar plates (wells 2 and 6) or diphasically (wells 3 and 5). The broth from an uninoculated culture gave no precipitin band (not shown).

**Cross-reactions between C. vaginale and possible related bacteria.** Anti-14018 Di antisera was subsequently employed in an attempt to demonstrate a common antigenic
FIG. 1. Demonstration of diffusible antigens of C. vaginale strain 14018. Anti-14018 Bld (A, center well) and anti-14018 Di (B, center well) were reacted against the extract of sonicated 14018 blood-grown cells (wells 1, 3, and 5) and the extract of sonicated 14018 diphasically grown cells (wells 2, 4, and 6). C, Line drawing of A.

FIG. 2. Effect of growth media upon the production of C. vaginale strain 14018 antigens. Anti-14018 Di (center well) was reacted against the broth extract of strain 14018 (wells 1 and 4), the extract of sonicated 14018 blood-grown cells (wells 2 and 6), and the extract of sonicated 14018 diphasically grown cells (wells 3 and 5).

determinant among 14 C. vaginale isolates. Although the number and intensity of bands varied, all C. vaginale isolates tested did share an antigenic determinant (Fig. 3). Bands of identity were visible between C. vaginale ATCC strain 14018 (well a) and C. vaginale isolates, 594, 8226, 6488 D, 6488 W, V23, V44, 359, 1544, 1575, 1637, 6234, and 8315 (wells 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, and 15, respectively). Spur formation indicative of partial identity appeared to be present with C. vaginale isolates T94 (well 1) and 144 (well 9). No bands formed with extracts of the vaginal diptheroid 8372 (well 16) or the negative control (well 3). A curious feature of this system is that the extract of C. vaginale strain 14018 Di was found to produce four visible distinct precipitin bands (Fig. 3, well a) with anti-14018 Di antiserum when the extract was placed in a well bounded by wells containing extracts of other C. vaginale isolates. When the extract was placed in a well
not bounded by wells containing extracts of other *C. vaginale* isolates, only one diffuse band was obtained (Fig. 4, well a).

Possible related bacteria were included in this study to determine the specificity of the Ouchterlony reactions (Fig. 4). The bacteria were chosen on the basis of morphology and common site of infection. Extracts of *C. cervicis* (13) (well 1), *C. xerosis* (7711) (well 2), *C. diphtheriae* (11913) (well 3), *L. acidophilus* (4356) (well 4), and vaginal diphtheroids 417 and 6649 (well 5 and 6, respectively) did not produce a precipitin band with anti-14018 Di antiserum. Therefore, this Ouchterlony analysis did not detect the existence of a common antigenic determinant between *C. vaginale* and strains representative of the genera *Corynebacterium* or *Lactobacillus*. No reaction was observed with the control, which consisted of an extract prepared from uninoculated diphasic medium (well 7).

**DISCUSSION**

The Ouchterlony technique was employed to examine the antigenic composition of *C. vaginale* as well as the effect of growth media upon the production of *C. vaginale* antigens. Although antisera prepared against *C. vaginale* type strain 14018 formed a varying number of precipitin bands (1 to 4) with *C. vaginale* type strains and clinical isolates, at least one band was found to be common to all of the *C. vaginale* isolates tested. A reaction of identity was observed between *C. vaginale* type strain 594 (ATCC no. 14018) and 12 of the *C. vaginale* isolates.

**Fig. 3.** Reactions between *C. vaginale* type strain 14018 and organisms which morphologically resemble *C. vaginale*. Center wells: (A) antiserum prepared against *C. vaginale* grown diphasically, (a) extract of 14018 sonicated cells grown diphasically. Outer wells: extracts of sonicated diphasically grown strains of *C. vaginale*; (1) T94, (2) 594, (3) negative control (an extract prepared from uninoculated diphasic media), (4) 8226, (5) 6488 D, (6) 6488 W, (7) V23, (8) V44, (9) 144, (10) 339, (11) 1544, (12) 1575, (13) 1637, (14) 6224, (15) 8315, (16) 8372.

**Fig. 4.** Reactions between *C. vaginale* type strain 14018 and heterologous bacteria. Center wells: (A) antiserum prepared against *C. vaginale* grown diphasically, (a) extract of 14018 sonicated cells diphasically grown. Outer wells: extracts of heterologous bacteria; (1) *C. cervicis*, (2) *C. xerosis*, (3) *C. diphtheriae*, (4) *L. acidophilus*, vaginal diphtheroid-like isolates (5) 417 and (6) 6659, (7) negative control (an extract prepared from uninoculated diphasic media).
Bands of partial identity appeared to form with C. vaginale type strain 594 (ATCC no. 14018) and two of the other clinical isolates.

It is believed that these precipitin bands contained more than one component. Because of the hazy flocculant nature of some bands and "band splitting" in certain systems, no exact enumeration of the number of bands could be made. Band splitting was produced only when wells containing extracts of the homologous system were bordered by wells containing extracts of related organisms. The cause of the band splitting is obscure at present, but a similar phenomenon has been found with other bacteria (1, 4). Possibly the heterologous strain possesses a different concentration of a specific antigenic determinant than does the homologous strain which (i) tends to enhance the formation of a band previously not visible or (ii) causes a band to split away from an apparent homogeneous band due to attractive forces. Further studies must be performed to reach a clear explanation.

In the present investigation, we were unable to demonstrate an antigenic relationship between C. vaginale and members of the genera Corynebacterium or Lactobacillus. In addition, no antigenic relationship could be detected between C. vaginale and vaginal diphtheroids, which morphologically could be confused with C. vaginale. These results are similar to those obtained in our previous studies utilizing the indirect fluorescent-antibody technique (7). Although low-titered fluorescent reactions occurred when anti-C. vaginale antiserum was tested against members of the genera Corynebacterium and Lactobacillus, adsorption of the homologous serum with these organisms did not noticeably decrease the fluorescent titer of the homologous system.

To determine whether there might be distinct antigenic differences between the organisms when grown diphasically or on blood, extracts of cells grown on both media were prepared by sonication and examined by the Ouchterlony technique. The extract of diphasically grown 14018 cells produced two precipitin bands with anti-14018 Bld, whereas the extract of the cells grown on blood agar plates formed only one precipitin band with this antiserum. However, both extracts produced two precipitin bands with anti-14018 Di antiserum. These results may be due to the fact that: (i) the diphasic medium either stimulates an increased production of a specific antigenic determinant present on C. vaginale cells and/or (ii) the antigen is more readily available for antigenic recognition when cells are grown on the diphasic medium.

Tests employing the indirect fluorescent-antibody technique have also revealed that antiserum prepared against C. vaginale strain 594 (ATCC no. 14018) grown diphasically reacted positively with all C. vaginale reference strains and clinical isolates. In contrast, antiserum prepared against the same strain grown on blood agar plates produced no fluorescence when reacted with the clinical isolates (7).

Studies were made of the diphasic broth in which C. vaginale organisms had been grown in order to determine whether diffusible antigens were produced. The extract of the broth in which C. vaginale strain 14018 was grown diphasically gave a single precipitin band when anti-14018 Di antiserum was reacted with the cell extracts of C. vaginale grown diphasically. The precipitin band also coalesced with the single band produced by cell extracts prepared from C. vaginale grown on blood agar plates. Thus, it appears that C. vaginale produced at least one antigen of high molecular weight that is released relatively easily from the cells into the medium. Work is presently in progress to determine whether this diffusible antigen is common to all of the C. vaginale isolates.

Therefore, Ouchterlony analysis, although time consuming, could be employed as a specific method for detecting strains of C. vaginale. However, the pitfalls of sonication, such as overheating the extracts, must be kept in mind. It is hoped that these studies will give some insight into the antigenic structure of C. vaginale and will lead to more detailed work that will answer the problems associated with the proper identification and taxonomic classification of this organism.

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