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Indirect Fluorescent-Antibody Method for the Identification of *Corynebacterium vaginale*

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The indirect fluorescent-antibody technique was employed in an attempt to develop a rapid method of identification of *Corynebacterium vaginale*. Six reference strains and ten clinical isolates selected on the basis of morphology and conventional biochemical tests were compared. Antisera were prepared in rabbits against the six reference strains. The most satisfactory antiserum was that prepared using strain 14018 grown diphasically (14018 Di) as the antigen. Certain of the antisera did exhibit a cross-reacting titer when reacted against *Corynebacterium diptheriae*, *Corynebacterium xerosis*, or *Lactobacillus acidophilus*. However, antisera adsorbed with these bacteria did not exhibit a significant decrease in titer when reacted against the homologous strain. Various other species of *Corynebacterium* as well as species of *Nocardia*, *Actinomyces*, *Hemophilus*, and *Streptococcus* did not fluoresce with the antisera. A specific antiserum was prepared by adsorbing anti-14018 Di with *L. acidophilus*. The adsorption removed the cross-reacting antibody but did not affect the staining reaction with *C. vaginale* strains. All reference strains and clinical isolates characterized as *C. vaginale* gave a definite positive reaction with the adsorbed anti-14018 Di. The specificity of the reactions was assessed by adsorbing the antiserum with the homologous strain. The data suggest that the indirect staining method will be of value in the rapid presumptive identification of *C. vaginale*.

Leopold (8) isolated an unclassified organism from cases of vaginitis and urethritis which he stated had characteristics in common with the genus *Hemophilus*. Independently, Gardner and Dukes (6) proposed the name *Hemophilus vaginalis* for the organism which they had also isolated from patients with vaginitis. Subsequent studies (2, 5, 7, 10, 12-15) have demonstrated that the organism differs morphologically and serologically from the genus *Hemophilus*. It has recently been proposed that the organism be designated *Corynebacterium vaginale* because of its Gram stain reaction, cellular morphology, and biochemical characteristics (3, 16).

Dunkelberg et al. (4) have described a method for the differentiation of *C. vaginale* from other *Corynebacterium* species and unclassified diphtheroid organisms. Colonies of *C. vaginale* have a typical round, domed, conical shape with a central button when grown on peptone-starch-dextrose (PSD) agar. Furthermore, *C. vaginale*, unlike other diphtheroid organisms, is inhibited by H_2O_2, lacks catalase, and ferments glucose, maltose, and starch.

This scheme, along with additional biochemical tests, was utilized in our laboratory to screen clinical specimens for possible *C. vaginale* isolates. However, these criteria are time consuming, lack standardization, and are quite vulnerable to error during laboratory manipulation. Hence it was decided to attempt the development of a more rapid system for the presumptive identification of *C. vaginale*.

The extensive literature on immunofluorescence in diagnostic microbiology and serology illustrates the significance of this technique in the clinical laboratory. This study was undertaken to determine whether the indirect fluorescent-antibody technique could be employed for the rapid presumptive identification of *C. vaginale*. Antisera were prepared against several type strains of *C. vaginale* and were tested against homologous and heterologous organisms. The specificity of the reaction was assessed by adsorption studies utilizing homologous and heterologous antigen controls. Individual differences were noted in the ability of the type strains to react with the same antiserum. It was possible to prepare an antiserum which

specifically reacted with 100% of the *C. vaginale* strains, suggesting that the indirect staining method will be of value in the rapid presumptive identification of *C. vaginale*.

MATERIALS AND METHODS

Organisms. W. E. Dunkelberg supplied five strains of *C. vaginale*: (i) 594 D (obtained from C. D. Dukes); (ii) 6488 D (obtained from R. E. Weaver); (iii) T94 (obtained from P. N. Edmunds); (iv) V28 and (v) V44 (two organisms isolated by W. E. Dunkelberg). R. E. Weaver forwarded strains 6488 W (isolated from a Bartholin gland) and 8226 (isolated from urine). P. Pease provided *Corynebacterium cervicis* strain 13. *H. vaginalis* (*C. vaginale*) strain 14018, *Corynebacterium xerosis* strain 7711, *Corynebacterium diphtheriae* strain 11913, *L. acidophilus* strain 4356, *Actinomyces bovis* strain 13683, *Nocardia asteroides* strain 19247 and *Hemophilus influenzae* strain 9247 were obtained from the American Type Culture Collection. *Corynebacterium hofmanii* strain 231 was obtained from the National Type Culture Collection. Eight organisms which morphologically and biochemically resembled *C. vaginale* as well as a strain of *Streptococcus mutans* which were isolated in our laboratory were also examined.

Media and tests. The isolation medium used was blood agar plates consisting of Trypticase soy agar (Difco) with 5% defribrinated sheep cells. The plates were incubated under increased carbon dioxide tension in a candle jar at 37 C. Although the organisms grew on the PSD agar devised by Dunkelberg and McVeigh (2), growth was more abundant on blood agar and contaminants were more readily observed.

Colony morphology of suspected *C. vaginale* strains was examined on the PSD agar using transmitted light.

The Sabouraud agar used in this study for the cultivation of *N. asteroides* was prepared commercially (BBL).

Tomato juice agar (BBL, prepared by instructions of manufacturer) was used in this study for the cultivation of *L. acidophilus*.

The media for fermenation tests was prepared as described by Dunkelberg et al. (4). Control tubes without carbohydrate were used with each test. The transfer broth used to inoculate the carbohydrate tubes consisted of Brewer thioglycolate (Difco) enriched with 0.5% rabbit serum. The fermentation media was inoculated with 3 to 5 drops of a 24- to 48-h transfer broth culture and stabbed at least four to five times.

Potassium tellurite medium was prepared by adding 0.01% potassium tellurite to PSD agar.

Inhibition by H_2O_2 was tested for by placing a drop of 3% H_2O_2 on a heavily inoculated PSD agar plate and, after 24 to 48 h, checking for inhibition of growth.

Catalase production was tested for by adding a drop of 3% H_2O_2 to a good growth of the organism on PSD agar and observing for evolution of bubbles.

The methyl red test was performed by adding 1 drop of methyl red indicator to 1 ml of a dense culture grown in starch peptone dextrose broth (PSD broth). The broth has the following formulation: proteose peptone no. 3 (Difco), 2.0%; soluble starch, 1.9%; dextrose, 0.2%; $Na_2HPO_4 \cdot 0.1\%$; and $Na_2H\ PO_4 \cdot H_2O$, 0.1%.

Indole production was observed by overlaying 1 ml of a dense culture of the organism grown in PSD broth with Kovacs Reagent (Harleco).

Urease production was tested for in a broth having the following formulation; proteose peptone no. 3, 2%; dextrose, 0.2%; urea broth concentrate (Difco), 10%.

To obtain large yields of *C. vaginale* for the immunological studies, a diphasic medium was utilized which consisted of a solid phase of PSD agar overlaid with thioglycolate broth. Seventy-five milliliters of PSD agar was poured into a 250-ml flask which was then stoppered, autoclaved, and allowed to solidify. One hundred milliliters of sterile Brewer thioglycolate (Difco) was added to the flasks.

Preparation of antigens. *C. vaginale* (14018) was inoculated onto blood agar plates and incubated at 37 C for 48 to 72 h. Bacteria were washed off the blood plates with 0.5% Formalin and incubated at 37 C for 72 h.

C. vaginale strains T94, 594 D, 14018, 8226, 6488 D, and 6488 W were grown in the diphasic medium at 37 C for 72 h. Broth was removed from the diphasic culture and centrifuged at 2,000 rpm for 15 min. The organisms were resuspended in 0.5% Formalin and incubated at 37 C for 72 h.

Bacteria were washed five times with 0.9% saline containing 0.025% Formalin and 0.01% sodium azide. The bacterial suspensions were adjusted to a density equivalent to that of a no. 7 McFarland standard for inoculations.

Immunization. New Zealand white rabbits weighing 2 to 2.5 kg were inoculated by the following schedule: day 1, 0.1 ml of the cell suspension incorporated in 0.1 ml of Freund complete adjuvant (Difco) was injected intradermally and subcutaneously into several sites of the footpads and back; day 7, 0.1 ml of the cell suspension incorporated in 0.1 ml of Freund complete adjuvant was injected intramuscularly into the thigh; and day 21, 1.0 ml of the cell suspension was injected intravenously into the ear. The rabbits were bled on day 28.

Indirect fluorescent-antibody staining. Bacteria grown either on blood agar plates or diphasically for 48 to 72 h were washed three times with 5 ml of normal saline. The sediments were resuspended in saline, and the turbidity was adjusted to that of a McFarland no. 3 standard. A loopful of organisms was spread on alcohol-washed slides, and the slides were allowed to air-dry. The slides were fixed with 95% ethanol for 1 min, washed in fluorescent treponemal antibody (FTA) hemagglutination buffer (pH 7.3) (BBL) for 5 min, and air-dried.

The smears were overlayed with an antiserum or a normal rabbit control serum and incubated in a moist chamber at 37 C for 30 min. Excess antiserum was removed by rinsing with the FTA hemagglutination buffer. Slides were then soaked in two changes of the FTA hemagglutination buffer (5 min), once in distilled water (5 min), and then air-dried. The appropriate dilution (the working dilution as described below) of fluorescein-conjugated goat anti-rabbit globulin

(BBL) was overlayed on the smears, and the slides were incubated at 37 C for 30 min. The smears were washed as described previously and air-dried, a drop of buffered glycerol-saline (pH 7.3) (BBL) was added, and the slides were prepared with a cover slip. Intensity of fluorescence was rated from 0 to 4+. Reactions of 2+ or greater were considered positive.

Determination of the working dilution of the goat anti-rabbit conjugate for use in the indirect method. Smears from *C. vaginale* strain 14018 Di (strain grown diphasically) were reacted with a 1:10 dilution of anti-*C. vaginale* 14018 Di antiserum and were then stained with 1:10 to 1:1,280 dilutions of goat anti-rabbit conjugate prepared in FTA buffer. These were examined, and the highest dilution which stained with 4+ fluorescent intensity was determined as the staining titer of the conjugate. The appropriate dilution of the conjugate for routine use, the working dilution, was arbitrarily chosen to be twice the concentration of the conjugate in the staining titer (e.g., if the staining titer was found to be 1:80, a 1:40 working dilution was used). The staining titer and the working dilution were determined for each lot of conjugate prior to use.

Serum titers. Titers of the antisera were determined by the standard doubling-dilutions method. Separate smears were overlayed with each of the twofold dilutions of serum and processed by the indirect fluorescent technique. The highest dilution of antiserum giving at least a 2+ fluorescent reaction was considered the titration end point.

Adsorptions. Bacteria grown for 48 to 72 h were washed three times with sterile saline. A 0.1-ml amount of packed, washed bacteria was mixed with 0.5 ml of antisera diluted 1:5 with saline. The mixture was incubated at 45 to 50 C for 2 h and overnight at 4 C. Adsorptions were repeated until a negative reaction occurred when the adsorbed antiserum was reacted with the adsorbing antigen.

Fluorescent microscopy. Microscopy was performed with an AO Spencer microscope equipped with an Osram HBO 200 high-pressure mercury lamp and a dark-field condenser for immersion oil. The following filter combinations were used: Corning 5970 in combination with a yellow barrier filter or a BG 12 in combination with a yellow orange barrier filter. The magnification used in microscopy was a 10× ocular lens and 45× objective lens.

RESULTS

Biochemical characteristics. The tested *C. vaginale* strains showed a high degree of similarity in their biochemical and cultural characteristics. Table 1 summarizes the results obtained when the six reference strains and ten clinical isolates which biochemically resembled *C. vaginale* were tested. Clinical isolates were chosen on the basis of Gram stain and colony morphology on blood agar plates. Nonhemolytic colonies measuring approximately 0.4 to 0.8 mm were visible in 36 to 48 h and when stained appeared as Gram-variable, diphtheroid-like organisms (Fig. 1). The Gram stain reaction observed was dependent upon the age of the culture, and variability was noted both in the staining reaction and morphology of the organism. The organisms in young (18 h) cultures contained a mixture of diphtheroid-like rods and gram-positive coccobacillary forms. As the age of the culture increased, gram negativity increased and coccobacillary forms grew diphtheroid-like with gram-positive beading. By 72 h only masses of gram-negative material were observed.

As shown in Table 1, all the *C. vaginale* strains fermented glucose, maltose, and starch, were nonhemolytic, were inhibited by H_2O_2, did not produce catalase, urease, or indole, produced a positive methyl red test, and did not reduce potassium tellurite. Arabinose was also fermented by six of the sixteen strains listed and xylose by 1 of the 16 strains. Mannitol was not fermented by any of the 16 strains.

The species of *Corynebacterium* tested exhibited a variety of biochemical reactions. All the *Corynebacterium* sp. tested produced catalase, reduced potassium tellurite, were inhibited by H_2O_2, were nonhemolytic and did not produce indole. *C. diphtheriae* strain 11913 fermented glucose, maltose, and starch, did not ferment arabinose, xylose, or mannitol, did not produce urease, and produced a negative methyl red test. *C. hofmanii* strain 231 and *C. cervicus* strain 13 did not ferment any of the carbohydrates tested. *C. hofmanii* did produce urease and gave a negative methyl red test. *C. cervicis* did not produce urease and gave a negative methyl red test.

Effect of fixation and culture media. The effect of fixation with acetone, ethanol, or heat on the staining reactions of blood-grown *Corynebacterium* sp. was examined. Staining after fixation in 95% ethanol for 1 min gave the most intense fluorescence. Acetone fixation for 1 min or gentle heating slightly decreased fluorescent intensity. Therefore, alcohol fixation was used throughout our work.

The reference strains grown on blood and diphasically were examined for variation in fluorescence intensity. Organisms grown on blood generally gave a somewhat greater intensity of fluorescence and the reactions were more consistent.

Fluorescent staining reactions of C. vaginale-type strains and clinical isolates biochemically resembling C. vaginale. Antisera were prepared against reference strains of *C. vaginale* grown diphasically (anti-T94 Di, anti-594 Di, anti-14018 Di, anti-8226 Di, anti-

TABLE 1. *Biochemical reactions of various Corynebacterium sp.*

Organism	PSD + carbohydrate[a]												
	Glucose	Maltose	Starch	Arabinose	Xylose	Mannitol	Hemolysis	H_2O_2 Inhibition	Catalase	Urease	Indole	Methyl red	Potassium tellurite
C. vaginale reference strains													
T94	A	A	A	–	–	–	NH	+	–	–	–	+	–
594	A	A	A	–	–	–	NH	+	–	–	–	+	–
14018	A	A	A	A	–	–	NH	+	–	–	–	+	–
8226	A	A	A	A	–	–	NH	+	–	–	–	+	–
6488 D	A	A	A	A	–	–	NH	+	–	–	–	+	–
6488 W	A	A	A	A	–	–	NH	+	–	–	–	+	–
C. vaginale clinical isolates													
V28	A	A	A	–	–	–	NH	+	–	–	–	+	–
V44	A	A	A	–	–	–	NH	+	–	–	–	+	–
144	A	A	A	–	–	–	NH	+	–	–	–	+	–
359	A	A	A	–	–	–	NH	+	–	–	–	+	–
1544	A	A	A	–	–	–	NH	+		–	–	+	–
1575	A	A	A	–	–	–	NH	+	–	–	–	+	–
1637	A	A	A	–	–	–	NH	+	–	–	–	+	–
6234	A	A	A	A	–	–	NH	+	–	–	–	+	–
8315	A	A	A	–	–	–	NH	+	–	–	–	+	–
8372	A	A	A	A	A	–	NH	+	–	–	–	–	–
Corynebacterium sp.													
C. diphtheriae (11913)	A	A	A	–	–	–	NH	+	+	–	–	+	R
C. xerosis (7711)	A	A	–	–	–	–	NH	+	+	–	–	–	R
C. hofmanii (231)	–	–	–	–	–	–	NH	+	+	+	–	–	R
C. cervicis (13)	–	–	–	–	–	–	NH	+	+	–	–	–	R

[a] A, Acid reaction; –, negative reaction; NH, no hemolysis; +, positive reaction; R, reduction.

FIG. 1. *Gram stain of C. vaginale after 36 h of growth in PSD-thioglycolate diphasic medium. Magnification of this photograph is approximately ×9,000.*

6488 D Di, and anti-6488 W Di) and one of the type strains grown on blood agar plates (anti-14018 Bld). Each of the anti-*C. vaginale* antisera was tested at an initial dilution of 1:10 to screen for reactivity against each of the reference strains and clinical isolates, and the results obtained when employing the indirect fluorescent-antibody staining technique are shown in Table 2. All reference strains fluoresced brightly after exposure to the homologous antiserum, but neither they nor the clinical isolates fluoresced after exposure to normal rabbit serum. Individual differences were noted in the capacity of the strains to react with the same antiserum. The antisera are listed from left to right in order of intensity of reactions and the number of positive reactions obtained. Three of the antisera were highly reactive: anti-14018 Di gave a positive reaction with 15 of the 16 organisms tested, and anti-8226 Di and

TABLE 2. *Indirect fluorescent staining reactions of C. vaginale reference strains and clinical isolates, using anti-C. vaginale antisera and goat anti-rabbit conjugate*[a]

Organism	Antisera (1:10 dilution)[b]							
	14108 Di	8226 Di	594 Di	6488 Di	T94 Di	6488 W Di	14018 Bld	NRS
Reference strains								
14018	3-4	3-4	3-4	2-3	3	1-2	4	0-1
6488 D	3	3-4	3-4	3-4	2	0-1	2-3	0-1
8226	2-3	3-4	3-4	3-4	1	0-1	2-3	0-1
594	3-4	3-4	3-4	1-2	3-4	1-2	4	0-1
T94	3-4	3-4	3-4	0-1	3-4	1-2	3-4	0-1
6488 W	3	3-4	3-4	3	1	3	0-1	0-1
Clinical isolates								
1575	3-4	3	3-4	2-3	3-4	2-3	3-4	0-1
V28	3-4	2-3	2-3	2-3	2-3	3-4	0-1	0-1
8315	2-3	3-4	3-4	2-3	3-4	1-2	0-1	0-1
1637	3-4	2-3	2-3	2	1-2	0-1	0-1	0-1
359	3	3	3	2	0	0-1	0-1	0-1
6234	2-3	3	3-4	0-1	1-2	0-1	0-1	0-1
V44	3-4	2-3	2-3	0-1	2-3	1-2	0-1	0-1
1544	3-4	2-3	2	0-1	1-2	1-2	0-1	0-1
144	3	1-2	1-2	0-1	1-2	0-1	0-1	0-1
8372	1-2	1-2	1-2	1-2	1-2	0-1	0-1	0-1

[a] Goat anti-rabbit conjugate used at a 1:20 dilution.

[b] Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity.

[c] Abbreviations: Di, Organism grown diphasically; Bld, organism grown on blood; NRS, normal rabbit serum.

anti-594 Di reacted with 14 of the 16 organisms tested. The remaining antisera were less reactive; anti-6488 Di reacted with four of the six reference strains and five of the ten clinical isolates; anti-T94 Di reacted with four of the six reference strains and four of the ten clinical isolates tested; anti-6488 W reacted only with the homologous type strain and two clinical isolates. It can be noted that anti-14018 Bld reacted differently than anti-14018 Di; i.e., anti-14018 Bld reacted with five of the six reference strains and only one of the clinical isolates.

Titers of the two most highly reactive anti-*C. vaginale* antisera (anti-594 Di and anti-14018 Di) against C. vaginale reference strains and clinical isolates generally ranged from 20 to 640. Since it was found that some clinical isolates reacted at a staining titer of 20, the anti-*C. vaginale* antiserum has subsequently been used routinely at a working dilution of 1:10 (the working dilution is arbitrarily chosen to be twice the concentration of the staining dilution).

Fluorescent staining reactions of heterologous bacteria. The reactivity of the anti-*C. vaginale* antisera with possible related organisms was assessed, and these reactions are shown in Table 3. The organisms tested (*L. acidophilus*, *C. diphtheriae*, *C. xerosis*, *C. hofmanii*, *H. influenzae*, *N. asteroides*, *A. bovis*, and *S. mutans*) were chosen on the basis of morphology and site of infection. Four of the seven antisera (anti-594 Di, anti-14018 Di, anti-8226 Di, and anti-14018 Bld) reacted with *L. acidophilus*. Two of the antisera (anti-8226 Di and anti-6488 D Di) reacted with *C. diphtheriae* and two of the antisera (anti-14018 Bld and anti-8226 Di) reacted with *C. xerosis*. Negative reactions were obtained after staining *C. cervices*, *C. hofmanii*, *H. influenzae*, *A. Bovis*, *N. asteroides*, and *S. mutans* with the seven antisera. Normal rabbit serum produced a negative reaction with all of the heterologous organisms tested.

When an apparent cross-reaction was noted, the titers of the cross-reactions were determined. Using the criteria that a positive reaction requires at least 2+ fluorescence, anti-14018 Bld gave a titer of 80 when reacted against *L. acidophilus* and *C. xerosis*. Each of the other antisera gave a titer of 10 when reacted against the heterologous organism.

To study further the apparent cross-reactions of anti-*C. vaginale* antisera with heterologous bacteria, all anti-*C. vaginale* antisera showing a reaction with a heterologous organism were adsorbed with the apparent cross-reacting organism and then the homologous titer was compared to that obtained before adsorption. Anti-

sera were adsorbed at least twice and a final adsorption was performed after a negative fluorescent reaction was obtained with the heterologous adsorbing organism (Table 4). There was no significant decrease in the titers of any of the anti-*C. vaginale* antisera against the homologous strain after adsorption with the apparent cross-reacting heterologous bacteria. The homologous titers after adsorption with the cells of the cross-reacting bacteria were generally decreased only one- or twofold, and the fluorescence to the cross-reacting bacteria was eliminated.

Development of clinical method of identification of C. vaginale. The previous results indicate that *C. diphtheriae*, *C. xerosis*, and *L. acidophilus* when present in clinical specimens might be expected to stain. Because they could not always be differentiated readily from *C. vaginale* on the basis of morphology, the antisera would need to be treated or used in some manner whereby reactions with heterologous bacteria could be eliminated.

Various techniques were utilized in an attempt to eliminate cross-reactions. An initial attempt was made to eliminate the problem of cross-reactions of *C. vaginale* antisera with heterologous organisms by simple dilution of the antisera. However, at a dilution at which all the heterologous reactions were eliminated (1:80), the reactions against the homologous organisms were often weak (2+) or borderline (1-2+).

Adsorptions were next undertaken in an attempt to eliminate the reactions of the anti-*C. vaginale* antisera with heterologous bacteria. Two of the most highly reactive antisera (anti-594 Di and anti-14018 Di) were adsorbed with the heterologous reacting organism (*L. acidophilus*). Adsorbing anti-594 Di and anti-14018 Di with *L. acidophilus* completely eliminated the apparent cross-reaction. Next, the adsorbed antisera were retested for their reactivity against all of the *C. vaginale* reference strains and clinical isolates, since an antiserum to be used in a rapid screening test for *C. vaginale* should react with all proven *C. vaginale* isolates. The adsorbed anti-594 Di reacted with all of the reference strains and seven of the ten clinical isolates tested (Table 5). Adsorbed anti-14018 Di reacted with all the organisms tested except for clinical isolate 8372. This clinical isolate has subsequently been shown not to be a *C. vaginale* isolate.

TABLE 4. *Homologous titers of C. vaginale antisera obtained before and after adsorption with heterologous cross-reacting bacteria as detected by the indirect fluorescent-antibody technique using goat anti-rabbit conjugate*[a]

Antiserum	Adsorbing bacteria	Titer before adsorption	Titer after adsorption
Anti-8226	*C. diphtheriae* (11913)	320[b]	320
Anti-6488 D Di[c]	*C. diphtheriae* (11913)	640	160
Anti-14018 Bld	*C. xerosis* (7711)	640	320
Anti-8226	*C. xerosis* (7711)	320	160
Anti-594 Di	*L. acidophilus* (4356)	640	160
Anti-14018 Di	*L. acidophilus* (4356)	320	320
Anti-8226 Di	*L. acidophilus* (4356)	320	320
Anti-14018 Bld	*L. acidophilus* (4356)	640	320

[a] Goat anti-rabbit conjugate used at a 1:20 dilution.

[b] Highest serum dilution resulting in at least a 2+ fluorescence.

[c] Abbreviations: Di, organism grown diphasically; Bld, organism grown on blood.

TABLE 3. *Indirect fluorescent staining reactions of various bacterial strains and species using anti-C. vaginale antisera and goat-anti rabbit conjugate*[a]

Organism tested	Antiserum (1:10 dilution) prepared against *C. vaginale* strain number[b]							
	T49 Di[c]	594 Di	14018 Bld	14018 Di	8226 Di	6488 D Di	6488 W Di	NRS
L. acidophilus (4356)	1-2	2-3	3-4	2	2-3	1-2	1-2	0-1
C. diphtheriae (11913)	1-2	1-2	0-1	0-1	0-1	0-1	0-1	0-1
C. xerosis (7711)	0-1	0-1	2-3	0-1	2	0-1	0-1	0-1
C. cervicis (13)	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
C. hofmanii (231)	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
H. influenzae (9247)	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
N. asteroides (19247)	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
A. bovis (13683)	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
S. mutans	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1

[a] Goat anti-rabbit conjugate used at a 1:20 dilution.

[b] Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity.

[c] Abbrevations: Di, organism grown diphasically; Bld, organism grown on blood agar plates; NRS, normal rabbit serum.

TABLE 5. *Indirect fluorescent staining reactions of anti-C. vaginale adsorbed with L. acidophilus versus C. vaginale reference strains and clinical isolates using goat anti-rabbit conjugate*[a]

Organism	Antisera[b]		
	anti-14018 Di	anti-594 Di	NRS
Reference strain			
14018	3-4[c]	3-4	0-1
594	3-4	3-4	0-1
6488 D	3-4	3-4	0-1
8226	3-4	2-3	0-1
T94	3	2-3	0-1
6488 W	2-3	2-3	0-1
Clinical isolates			
8315	3	3-4	0-1
1575	3	3	0-1
V28	2-3	2-3	0-1
V44	2-3	2-3	0-1
1637	3	2	0-1
349	2-3	2	0-1
6234	2-3	2	0-1
1544	2-3	1-2	0-1
144	2	0-1	0-1
8372	0-1	0-1	0-1
L. acidophilus	0-1	0-1	0-1

[a] Goat anti-rabbit conjugate used at a 1:20 dilution.

[b] Abbreviations: Di, organism grown diphasically; NRS, normal rabbit serum.

[c] Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity.

One further test of specificity was performed in which anti-14018 Di was adsorbed with the homologous organism. This adsorbed antiserum was tested against the reference strains and clinical isolates, and no fluorescence was observed.

DISCUSSION

It was decided to attempt to identify *C. vaginale* by means of serological techniques. Two major difficulties occur when identifying *C. vaginale* on the basis of serological procedures. The organism is frequently very rough and thus agglutination tests cannot be done, or growth is usually so scanty that insufficient antigen is available. Although the development of the diphasic medium provided abundant growth of the organism, we were unable to eliminate the roughness of the organism.

Since these difficulties can generally be overcome by employing fluorescent microscopy, an attempt was made to develop a fluorescent-antibody technique for the rapid presumptive identification of *C. vaginale*.

Antisera were prepared in rabbits against the six reference strains of *C. vaginale* and were tested against homologous and heterologous organisms by the indirect fluorescent-antibody technique. Differences in reactivity against the type strains were noted among the antisera; three of the antisera (anti-14018 Di, anti-594 Di, and anti-8226) were highly reactive and produced a positive reaction with all the type strains, whereas the remaining four antisera (anti-14018 Bld, anti-6488W Di, and anti-T94 Di) did not react with all of the type strains. Differences were also noted in antisera prepared against the same strain obtained from two different sources. For example, antiserum prepared against 6488 W, which was obtained from R. E. Weaver, reacted only with two of the seven *C. vaginale* clinical isolates tested and none of the reference strains. In contrast, antiserum prepared against 6488 D, supposedly the same strain but obtained from W. E. Dunkelberg, reacted with nine of the fifteen isolates tested. These variations may be accounted for by (i) differences in amount of antigen present, (ii) lack of a specific antigen in some strains, (iii) slight mutations during transfer, or (iv) differences in antibody response of the rabbits. Previous investigators have observed that the immunological response of various rabbits to antigen may be quite different (11).

It was noted that apparent nonreciprocal cross-reactions occurred when certain of the antisera were reacted with the reference strains. For example, anti-594 reacted with all the type strains but anti-6488 D and anti-6488 W did not react with 594. In addition, anti-8226 reacted with five of the type strains including T94. However, anti-T94 did not react with 8226. It is difficult to advance a definitive explanation for this phenomenon. There was little possibility of contamination of the 594 antigen cells with cells of strain 6488 D or 6488 W, nor 8226 antigen cells with cells of strain T94. Dudman (1) observed a similar nonreciprocal reaction between strains of *Rhizobium japonicum*. He concluded that either (i) cross-reacting antibodies were involved or (ii) the antigenic determinant is present but in a limited amount.

The type of culture medium on which the organism is grown may also play an important role in the reaction observed. Antiserum prepared against 14018 grown on blood reacted with five of the six reference strains and only one of the clinical isolates, whereas antiserum prepared against 14018 grown diphasically reacted with all strains of *C. vaginale* tested. Previous investigators have demonstrated variations in fluorescent intensity dependent upon

medium employed (9). Additional evidence that the type of medium employed plays a major role in the type of antiserum prepared has been obtained by Ouchterlony analysis, which shows that organisms grown diphasically have an additional precipitin band when compared to the same organism grown on blood (manuscript in preparation). These results may be due to the fact that (i) the diphasic media stimulates an increased production of a specific antigenic determinant present on *C. vaginale* cells and/or (ii) when the cells are grown in the diphasic media the antigen is more readily available for antigenic recognition in the rabbit.

Several reports in the literature have indicated that problems of cross-reactions with heterologous bacteria occur in the development of a specific fluorescent-antibody identification technique. Therefore, various bacteria chosen on the basis of morphology and site of infection were included in this study. Two species of *Corynebacterium* as well as species of *Actinomyces*, *Nocardia*, *Hemophilus*, and *Streptococci* were fluoresced with each *C. vaginale* antiserum and were negative. Cross-reactions did occur with *C. diphtheriae*, *C. xerosis*, and *L. acidophilus*. Generally, titers of 10 or under were obtained with the heterologous cross-reacting bacteria. Two antisera did give titers of 80 with the heterologous species; i.e., anti-14018 Bld when reacted with *C. xerosis* or *L. acidophilus*, and anti-8226 Di when reacted with *C. xerosis*. However, after adsorbing the antisera with the heterologous bacteria, homologous titers were decreased only one- or twofold.

These apparent cross-reactions could be a source of error if interpretation of the tests was not made with great care. Therefore, to develop a specific indirect fluorescent method for identification of *C. vaginale*, various techniques were utilized in an attempt to eliminate the nonspecific cross-reactions. Diluting the antisera reduced intensity of heterologous fluorescent-antibody staining but did not completely eliminate borderline reactions. Diluting the antisera, furthermore, reduced homologous activity.

However, it was possible to selectively remove the responsible cross-reacting factors by adsorption. The two most highly reactive antisera (anti-14018 Di and anti-594 Di) were adsorbed with the cross-reacting heterologous organism (*L. acidophilus*) and were again tested for their ability to react with the reference strains and clinical isolates. Adsorbed anti-594 Di retained its ability to stain all reference strains, but lost its ability to stain two of the *C. vaginale* isolates. Adsorbed anti-14018 Di retained its ability to react with all of the reference strains and clinical isolates, whereas heterologous organisms exhibited only a 0 to 1+ fluorescence. The specificity of the reaction of anti-14018 Di with other *C. vaginale* reference strains and clinical isolates was further tested after adsorption of anti-14018 Di with the homologous strain. When this adsorbed antisera was reacted with the *C. vaginale* reference strains and clinical isolates, no fluorescence was observed. Hence, anti-14018 was chosen for use in our laboratory for the rapid presumptive identification of *C. vaginale*, since this antiserum was shown to react specifically with all of the reference strains and clinical isolates.

Further evaluation studies in this lab have demonstrated that indirect fluorescent microscopy is as specific and sensitive as the conventional biochemical tests, and is also a more rapid presumptive method of identification. It is hoped that our studies will provide a basis for workers to further attempt to investigate the intriguing problem of identification and classification of *C. vaginale*.

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