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Serological Methods for the Identification of Corynebacterium vaginale

Mary Frances Smaron
Loyola University Chicago

SEROLOGICAL METHODS FOR THE IDENTIFICATION OF CORYNEBACTERIUM VAGINALE

by

Mary Fran Smaron

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I. INTRODUCTION

Corynebacterium vaginale (*C. vaginale*)* is a fastidious gram variable pleomorphic bacillus. Extensive literature has been published concerning the pathogenicity, taxonomy, and clinical identification of this organism. The purpose of this thesis was to study the antigenic structure of *C. vaginale* and possible related bacteria and to develop a simple, rapid method of identification of *C. vaginale*.

A. PATHOGENICITY

During the past three decades, bacteriologists and gynecologists have directed their attention toward elucidating the cause(s) of non-specific vaginitis. It was because of this quest that research on *C. vaginale* was first begun.

Leopold (26) isolated an "Hemophilus-like" organism in 16 of 58 vaginitis cases. Independently, Gardner and Dukes (21) isolated a similar organism from 12% of 1181 patients with vaginitis. Gardner and Dukes first attempted to establish the organism as a specific etiological agent of vaginitis. They claimed to have fulfilled Koch's postulates. Koch's first postulate states that the bacterium must be observed in every case of the disease. Ninety-two per cent of the patients

*This organism was originally referred to as *Hemophilus vaginalis* but subsequent biochemical and serological tests have caused several workers to designate this organism *C. vaginale*. Our work has shown that this organism more closely resembles the genus *Corynebacterium*, and therefore the organism will be referred to as *C. vaginale* throughout the thesis.

with a primary diagnosis of bacterial vaginitis were found to have C. vaginale infections. Koch's second postulate states that the bacterium must be isolated and grown in pure culture. In the 141 cases of vaginitis examined, C. vaginale was the only bacterium isolated in 85 cases and the predomonant organism in 56 cases. Koch's third postulate states that when the bacterium is inoculated into a susceptible animal, the disease should ensue. Thirteen human volunteers were infected with C. vaginale: ten neither developed the disease nor gave positive cultures, two gave positive cultures but exhibited no disease symptoms, one patient developed the disease and the organism was isolated in pure culture from her. This last patient fulfilled Koch's fourth postulate which states that the organism must be isolated from the infected animal.

Gardner and Dukes (21) reported that the organism is harbored by the male and is capable of being transmitted by sexual intercourse. They further stated that apparently C. vaginale is a "surface parasite without invasive tendencies." In a later study (20) which involved the investigation of 2, 251 office patients and 893 clinical patients, C. vaginale was diagnosed as the causative agent of bacterial vaginitis in 94.5% of the private patients and 84% of the clinical patients. On the basis of these studies Gardner and Dukes (20) concluded that C. vaginale is a frequent inhabitant of the female genital tract and is the pathogen responsible for the majority of "non-specific" urethritis.

Subsequent workers have similarly implicated C. vaginale as the causative agent in genital tract disorders. Lutz, Wurch, and Grotten (27) cultured C. vaginale in 20% of their patients with vaginitis. Brewer, Halpern, and Thomas (3) showed the organism in pure or nearly pure culture in 89 (42%) of 211 patients with leukorrhea.

However, other workers do not agree that C. vaginale is the cause of a specific disorder. Doell (7) at Wuerzburg, Germany was unable to culture C. vaginale from any of 300 female patients. Butler and Beakley (4) maintained that the absence of L. acidophilus caused vaginitis. Heltaii and Taleghany (24) examined 90 patients with vaginal infections, 30% of which were positive for C. vaginale. Twelve of these patients had C. vaginale only after sulfa therapy and eight of these were asymptomatic. They concluded, therefore, that C. vaginale is not a cause of vaginitis.

Still other researchers (11, 12) believe that C. vaginale may be part of the normal flora but represents an opportunistic pathogen of low virulence. Development of infection may then be dependent upon still undetermined alterations in the normal environment of the vaginal tract.

In summary, the pathogenicity of the organism is still questionable. The organism has been isolated in diseased patients but also in asymptomatic patients. This situation may be compared to that of Candida albicans which are normal flora in the genital tract and only produces disease under

certain conditions, such as following antibiotic therapy (20). Definite proof that C. vaginale is a pathogen awaits further investigation.

B. TAXONOMY

As the possible pathogenicity of C. vaginale became recognized, researchers attempted to determine the taxonomic position of the organism. When Leopold (26) isolated the organism, he believed that it had characteristics typical of the genus Hemophilus. He described it as a gram negative, non-motile, non-encapsulated, pleomorphic rod, 0.5 um wide and from 0.5 to 2 um long which appeared as a tiny, colorless, definitely hemolytic colony on Casman's blood agar after 48 hr. Independently, Gardner and Dukes (21) published similar microscopic and colony morphology data and proposed the name Hemophilus vaginalis (H. vaginalis).

1. Morphological and biochemical characteristics.

Much of the early work concentrated on the morphology, growth requirements and biochemical reactions of C. vaginale.

a. Evidence supporting the designation Hemophilus.

Amies and Jones (1) found the coccobacillary morphology of C. vaginale indistinguishable from Hemophilus aegypticus. They obtained no growth on blood agar but C. vaginale would grow on chocolate agar or medium containing serum and V factor. They suggested that the pleuropneumonia-like organisms found in genito-urinary infections were L forms of C. vaginale.

Gardner and Dukes (21) also suggested that C. vaginale requires serum and other accessory growth factors. Although the organism grew both aerobically and anaerobically, they obtained the most luxuriant growth on 10% sheep blood agar plates incubated in a candle jar. They also noted that 0.1% starch aided growth. However, they stressed that under minimal nutrient conditions X (hemin) and V (nicotinamide-adenine-dinucleotide) factors were needed for growth.

Tarlinton and D'Abrera (36) investigated fourteen strains of an organism which they had isolated in their laboratory from patients with vaginitis. These isolates were gram negative coccobacilli and V factor was essential for growth. They agreed with Gardner and Duke's identification of this organism as H. vaginalis.

b. Evidence supporting a designation other than Hemophilus.

The first evidence that the organism is not a member of the genus Hemophilus came from the studies of LaPage (25). He examined 37 clinical isolates resembling C. vaginale; 11 C. vaginale reference strains obtained from Gardner and Dukes, and Amies and Jones; and various species and strains of the genus Hemophilus. He divided these organisms into two main groups: "Hemophilus vaginalis-like" and Hemophilus influenzae-like" which encompassed the Amies and Jones isolates. The Hemophilus vaginalis group differed from the Hemophilus influenzae group in that they did not need X and V factors,

they grew on Casman's medium without blood, there was an absence of filaments, they were oxidase negative, and failed to grow on Fildes medium. The author suggested that further characterization of the organism should be done with emphasis on criteria representative of the genus Corynebacterium and Lactobacillus.

Subsequent reports were published on the tendency of cultures of the organism to stain gram positive (27, 3, 17, 10, 23). Edmunds (18) stated that this fact along with the lack of X and V requirements were the main objections to the organism being a species of Hemophilus. The organism grew at pH 4.5 and the most readily fermented carbohydrate was starch; two characteristics of the genus Lactobacillus. In fact, Amies and Garabedian (2) considered it a dissociated Lactobacillus.

Edmunds (18) as well as Brewer, Halperin and Thomas (3), also noted that the morphology of C. vaginale resembled that of the Corynebacterium. However, Edmunds (18) reported that the organism was definitely catalase negative and therefore not a Corynebacterium. He suggested it may represent a new genus.

Park (30) was able to get a luxuriant growth on a glucose starch agar. Because of its independence of X and V factor, he concluded it was misplaced in the genus Hemophilus. However, he considered the organism gram negative and should not be classified as a Corynebacterium. He also recommended the establishment of new genus.

Zinneman and Turner (38) emphasize that the main criterion for inclusion of an organism into the genus Hemophilus is its

dependence on either X or V factors or both. They investigated two strains obtained from Dukes and four obtained from Edmunds, and found that the organisms were gram positive on optimal media, and formed polar granules and "chinese characters." According to Zinneman and Turner (38) the organisms did not appear to have an absolute requirement for either X and/or V factor. Although the organisms did not produce catalase, they recommended the name C. vaginale since not all of the recognized species of Corynebacterium have this characteristic.

Dunkelberg and others have attempted to establish a relationship between C. vaginale and other members of Corynebacterium. Dunkelberg and McVeigh (13) determined the growth requirements of C. vaginale. They grew the organism in a semi-defined medium containing enzymatically hydrolyzed vitamin-free casein, inorganic salts (K_2HPO_4, KH_2PO_4, $MgSO_4$, H_2O, NaCl, $(NH_4)_2\ SO_4$), carbohydrates (glucose and maltose) and six purine and pyrimidine bases (adenine sulfate, guanine HCl, cytosine, uracil, thymine and xanthine) and found it required the following B-vitamins: thiamine HCl, riboflavin, niacin (or niacinamide), pteroylglutamic acid, and biotin. Since C. vaginale did not require X and/or V factor or an otherwise definable coenzyme-like substance, Dunkelberg and McVeigh thought it did not qualify as a member of the genus Hemophilus.

Dunkelberg and Moss (14) employed gas chromatography to study C. vaginale. They determined that acetic acid was the major volatile acid produced by C. vaginale strain 594. Because of the absence of proprionate, butyrate and branched-

chain fatty acids, they concluded C. vaginale should not be a member of the genera Proprionibacterium or Butyribacterium.

Dunkelberg, Skaggs, and Kellogg (15) studied various strains obtained from Dukes, Edmunds and Weaver. The organisms grew on 0.01% potassium tellurite medium, in 0.01% potassium cyanide and displayed metachromatic areas when stained by Alberts method. These are characteristics common to the genus Corynebacterium.

2. Cell wall analysis

Several investigators have examined the cell wall of C. vaginale as a possible taxonomic key for the elucidation of C. vaginale classification. Vickerstaff and Cole (37) analyzed the cell wall components of several strains of C. vaginale and related bacteria. The cell wall of one strain obtained from Edmunds was chemically similar to the genus Corynebacterium. Corynebacterium possesses the monosaccharides galactose and arabinose; and diaminopimelic acid, alanine and glutamic acid are the major amino acids. The NCTC (10287) strain had a cell wall similar to Actinomyces bovis. Diaminopimelic acid was lacking and both organisms possessed galactose, mannose, rhamnose and six deoxytalose. Two strains of C. vaginale, one isolated by Amies and Jones and the other obtained from Edmunds, possessed cell walls similar to the genus Hemophilus. Galactose was the major monosaccharide and a complex amino acid composition including diaminopimelic acid was found. Furthermore, these strains required X and V factors. Two strains

submitted by Edmunds could not be identified with any of the recognized genera examined. Various biochemical reactions were also performed but no specific pattern was recognized for any group. Vickerstaff and Cole (37) concluded that they were dealing with a heterogenous group of organisms and that certain strains resembled the genus Hemophilus, Corynebacterium or Actinomyces; while other strains did not resemble any of the genera examined.

Criswell et al. (5) employed electron microscopy and biochemical techniques to study C. vaginale strain 594 isolated by Dukes and Gardner. They found C. vaginale possessed a multiple layered cell wall, containing eleven to fourteen amino acids, a low mucopeptide content and no teichoic acid. In contrast, gram positive organisms generally have a small number of amino acids in their walls, a large percentage of mucopeptide and noticeable quantities of teichoic acids.

Criswell et al. (6) reinterated that C. vaginale possessed a trilaminar cell wall. They also found convulated and simple mesosomes, a fibrillar nucleoid and ribosomes. They explained that the pleomorphism of C. vaginale was due to multiple cross wall formations and polar enlargements. They agreed with Gardner and Dukes that the organism is a member of the genus Hemophilus.

Reyn et al. (35) had previously employed electron microscopy to study thin sections of the same strain of C. vaginale studied by Criswell et al. (5, 6) in addition to

Butyribacterium rettgeri, Corynebacterium diphtheriae, Lactobacillus acidophilus, Hemophilus influenzae and Neiserriae haemolysans. They concluded that the fine structure of C. vaginale, particularly its cell wall and septa, closely resembled gram positive organisms rather than gram negative organisms. The gram positivity of C. vaginale was further revealed in lysing cells; these cells did not swell until the cell wall was broken. In contrast, Hemophilus influenzae lost its original form without obvious lesions of the cell wall. Hemophilus influenzae had a typical three layered cell wall; whorled mesosomes were not found. Division occurred by simple constriction, where as division of C. vaginale was preceded by septum formation.

Reyn (35) found the structure of Lactobacillus acidophilus very different from that of C. vaginale. The cell wall of Lactobacillus acidophilus was much thicker than that of C. vaginale, the nuclear material of Lactobacillus acidophilus was distributed throughout the cytoplasm and division occurred in a slightly different manner in the two organisms.

3. Serological studies

A few investigators have studied the antigenic structure in an attempt to clarify the taxonomy of C. vaginale. Gardner and Dukes (22) searched for a common antigen present in C. vaginale isolates and the genus Hemophilus. They found no cross-agglutination between C. vaginale and three strains of the genus Hemophilus.

Peace and Laughton (32) employed the Ouchterlony technique to examine species of Hemophilus, strains of Corynebacterium cervicis and strains of C. vaginale isolated by Amies and Jones, Gardner and Dukes, and Edmunds. They found a common antigen between the GP2 strain of C. vaginale and Hemophilus influenzae. Furthermore, Hemophilus influenzae shared a common antigen with one of the Corynebacterium cervicis strains examined. Therefore, they concluded that a relationship exists between these bacteria. These results must be reinterpreted because other workers have noted that the GP2 strain actually contains two different bacteria: one organism resembles the genus Hemophilus and the other organism resembles the genus Corynebacterium.

Redmond and Kotcher (33) employed fluorescent microscopy and agglutination reactions and obtained different reactions with the various C. vaginale strains studied. Two strains obtained from Amies fluoresced and agglutinated with anti-Hemophilus aegypticus serum. There was no reaction between anti-Hemophilus aegypticus serum and various strains obtained from Dukes, Edmunds, King and organisms isolated in their laboratory. Five ATCC strains did fluoresce with anti-Hemophilus aegypticus but no agglutination occurred.

In conclusion, many investigators have pointed out the heterogeneity of the group of organisms designated C. vaginale and it is generally recognized that a specific set of criteria must be established for the identification of C. vaginale.

This would climinate much confusion in research and clinical laboratories.

C. ISOLATION AND IDENTIFICATION

1. Presence of "clue cells"

Gardner and Dukes (21) gave special attention to the diagnostic character of "clue cells" which they claimed are generally present in vaginal infections caused by C. vaginale. "Clue cells" are epithelial cells containing many gram negative bacilli either intracellularly or attached to their surface. Dunkelberg and Bosman (11) noted that Lactobacillus,while larger than C. vaginale,can often be found packed in or on epithelial cells, causing confusion with the genuine clue cells. They recommended using 95% ethyl alcohol as the decolorizing agent for the gram stain, since it would decolorize C. vaginale but not Lactobacillus whereas the use of a 4:1 alcohol: acetone mixture decolorized both organisms.

2. Biochemical criteria

Park (30) found that colony morphology on chocolate agar, gram stain reaction and growth inhibition by alpha hemolytic streptococci or pneumococci served as reliable criteria for identification.

Several investigators (38, 21, 18, 36) have studied fermentation reaction of the organisms. Maltose, glucose, and starch were among the carbohydrates consistently fermented. However, Tarlington (36) pointed out that many technical problems had to be overcome before reliable and consistant

results could be obtained.

Dunkelberg et al. (15) proposed a specific method for isolation and identification of C. vaginale. The principal features of this method are determination of fermentation reactions without blood or serum interference and employment of a clear medium to allow observation of colony morphology by transmitted light. Colonies of C. vaginale have a typical round domed, conical shape with a central button on the clear peptone-starch-dextrose agar. The method differentiates C. vaginale from unclassified genital diphtheroids. Wet mount examinations of genital discharge diphtheroids for clue cells, gram stain reaction, cell and colony morphology, growth inhibition by H_2O_2, lack of catalase, and fermentation of glucose, maltose and starch are the determinant factors.

3. Previous immunological studies

Several investigators attempted to establish an agglutination or precipitation technique but found that the organism after primary isolation would flocculate spontaneously (1, 22, 18). Edmunds (18) noted that complement fixation was impractical because large quantities of antigen would need to be standardized. He found that most strains agglutinated human red blood cells, but some agglutinated horse, ox or fowl cells. The agglutination was not due to fimbriae because mannose did not inhibit the reaction. However, he established no specific identification criteria.

Redmond and Kotcher (33) said spontaneous agglutination

could be eliminated by dispersing the organism in Hank's balanced salt solution and bovine albumin. Eight reference strains of C. vaginale gave agglutination titers ranging from 320 to 1280 with anti C. vaginale serum. Indirect immunofluorescence was also employed and titers ranged from 10 to 40. Therefore, Redmond and Kotcher concluded that a common antigen existed in various strains of C. vaginale.

4. Purpose of this research

The scheme described by Dunkelberg et al. (15) along with additional biochemical tests was utilized to screen clinical specimens for possible C. vaginale isolates. However, the techniques are time consuming, lack standardization and are quite vulnerable to error during laboratory manipulation. Hence we decided to attempt the development of a more rapid system for the identification of C. vaginale.

The extensive literature on immunofluorescence illustrates the significance of this technique in research and clinical laboratories. This study was performed to determine whether the indirect fluorescent antibody technique could be employed for the rapid identification of C. vaginale.

Antisera were prepared against several strains of C. vaginale. The antisera 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 all strains of C. vaginale tested suggesting that the indirect staining method will be of value in the rapid identification of C. vaginale.

The immunodiffusion study was undertaken to investigate the antigenic relationship among various strains of C. vaginale and possible related bacteria. Although fluorescent microscopy is a rapid sensitive technique, it lacks the resolving power of the immunodiffusion technique which has the ability to recognize numerous antigens and antibodies in a mixture and establish their relatedness. The antiserum shown to fluoresce with all reference and clinical isolates was utilized in the Ouchterlony studies. Sonicated cells were used as the source of antigen, thereby making available both extra cellular and intra cellular antigens to the system and increasing the chances of making fine distinctions.

All C. vaginale strains examined produced bands of identity or partial identity whereas no such bands were produced with heterologous bacteria. The present study also shows that the type of medium employed does play a major role in the production of a specific antigen by C. vaginale.

Our aim was to alleviate the problem of identification of C. vaginale and to further characterize this organism.

II. MATERIALS AND METHODS

Organisms. Five strains of C. vaginale: (1) 594 D (obtained from Dr. C. D. Dukes): (2) 6488 D (obtained from Dr. R. E. Weaver); (3) T94 (obtained from Dr. P. N. Edmunds); (4) V28 and (5) V44 (two organisms isolated by Dr. Dunkelberg) were supplied by Dr. W. E. Dunkelberg. Strains 6488 W (isolated from a Bartholin gland) and 8226 (isolated from urine) were forwarded by Dr. R. E. Weaver. Corynebacterium cervicis (C. cervicis) strain 13 was provided by Dr. P. Pease. Hemophilus vaginalis (C. vaginale) strain 14018, Corynebacterium xerosis (C. xerosis) strain 7711, Corynebacterium diphtheriae (C. diphtheriae) strain 11913, Lactobacillus acidophilus (L. acidophilus) strain 4356, Actinomyces bovis strain 13683 and Nocardia asteroides strain 19247 were obtained from the American Type Culture Collection. Corynebacterium hofmanii (C. hofmanii) strain 231 was obtained from the National Type Culture Collection. Ten vaginal diptheroid - like organisms isolated in our laboratory were also examined: seven of the isolates morphologically and biochemically resembled C. vaginale (144, 359, 1544, 1575, 1637, 6234, and 8315) and three of the isolates were biochemically unlike C. vaginale (8372, 317, and 6659).

Media and tests. The isolation medium used was blood agar plates consisting of trypticase soy agar (Difco, Detroit, Michigan) with 5% defibrinated sheep blood cells (Obine

Laboratories, Chicago, Illinois). 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 (13) growth was more abundant on blood agar and contaminants were more readily observed.

In order to obtain large yields of C. vaginale, a diphasic medium was utilized which consisted of a solid phase of PSD agar overlayered with thioglycollate broth. Seventy-five ml of PSD agar was poured into a 250 ml flask which was then stoppered, autoclaved and allowed to solidify. One hundred ml of sterile Brewer's thioglycollate (Difco, Detroit, Michigan) was added to the flasks. Flasks were incubated at 37 C for 48 to 72 hr.

Colony morphology of suspected C. vaginale strains was examined on the PSD agar using transmitted light. (American Optical co., Buffalo, New York).

The Sabouraud's agar used in this study for the cultivation of Nocardia asteroides was prepared commercially (BBL, Cockeysville, Maryland).

Tomato juice agar (BBL, Cockeysville, Maryland) was used in this study for the cultivation of L. acidophilus. When large yields of the organism were needed, a diphasic medium consisting of eugon broth and eugon agar was utilized. The incubation time, amounts of media and method of preparation were the same as described for the diphasic medium used to grow C. vaginale.

The medium for fermentation tests was prepared as described by Dunkelberg and McVeigh (13). Control tubes without carbohydrate were used with each test. The transfer broth used to inoculate the carbohydrate tubes consisted of Brewer's thioglycollate (Difco, Detroit, Michigan) enriched with 0.5% rabbit serum. The fermentation medium was inoculated with 3 to 5 drops of a 24 to 48 hr transfer broth culture and stabbed at least 4 to 5 times.

Potassium tellurite medium was prepared by adding 0.01% potassium tellurite (Difco, Detroit, Michigan) to PSD agar (13).

Inhibition by H_2O_2 was tested for by placing a drop of 3% H_2O_2 (Mallinckrot Chemical Works, St. Louis, Missouri) on a heavily inoculated PSD agar plate and after 24 to 48 hr, checking for inhibition of growth (13).

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 (13).

The Methyl Red test was performed by adding 1 drop of methyl red indicator to 1 ml of a dense culture grown in Proteose Peptone No. 3 broth (PSD broth). The broth has the following formulation: Difco Proteose Peptone No. 3, 2.0%; Soluble Starch, 1.0% glucose, 0.2%; Na_2HPO_4, 0.1% and $NaH_2PO_4 \cdot H_2O$, 0.1% (13).

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

Reagent (Harleco, Philadelphia, Penn.).

Urease production was determined in a broth having the following formulation; Difco Protecse Peptone No. 3, 2%; dextrose, 0.2% Difco urea broth concentrate, 10%.

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

C. vaginale strains T94, 594 D, 14018, 8226, 6488 D and 6488 W were grown in the diphasic medium at 37 C for 72 hr. Broth was removed from the diphasic culture and centrifuged at 2000 rev/min for 15 min. The organisms were resuspended in 0.5% formalin and incubated at 37 C for 72 hr.

Bacteria were washed five times with 0.9% saline containing 0.025% formalin and 0.01% sodium azide. McFarland's standards (28) were utilized to prepare suspensions containing approximately 2×10^9 organisms per ml for inoculations.

Immunization. New Zealand White rabbits (Scientific Small Animals, Arlington, Hts., Illinois) weighing 2-2.5 kg were inoculated by the following schedule: day 1, 0.1 ml of the 2×10^9 cell suspension incorporated in 0.1 ml Freund's complete adjuvant (Difco, Detroit, Michigan) was injected intradermally and subcutaneously into several sites of the foot pads and back; day 7, 0.1 ml of the cell suspension incorporated in 0.1 Freund's complete adjuvant was injected intramuscularly into the thigh; and day 21, 1.0 ml of the cell suspension

alone 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 hr were washed with saline, a loopful spread on alcohol washed slides and the slides allowed to air dry. The slides were fixed with 95% ethanol for 1 min, washed in FTA Hemagglutination buffer (BBL, Cockeysville, Maryland) for 5 min and air dried. The smears were overlayed with an antiserum or a normal rabbit serum control and incubated in a moist chamber at 37 C for 30 min. Excess antiserum was removed by rinsing the slide 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 air dried. Fluorescein-conjugated goat anti-rabbit globulin (BBL, Cockeysville, Maryland) was overlayed on the smears and the slides were incubated at 37 C for 30 min. The smears were washed as described previously, air dried, a drop of buffered glycerol-saline (BBL, Cockeysville, Maryland) was added and the slides were mounted with a coverslip. Intensity of fluorescence was rated from 0 to 4+. Reactions of 2+ or greater were considered positive.

Serum titers. Titers of the antisera were determined by the standard doubling-dilutions method. Separate smears were overlayed with each of the two-fold dilutions of serum and incubated in a moist chamber at 37 C for 30 min. Following

incubation, the cells were washed two times in FTA Hemagglutination buffer, once in distilled water, and air dried. Fluorescein-conjugated goat anti-human gamma globulin was added to the slides and they were again incubated at 37 C for 30 min in a moist chamber. They were subsequently mounted and examined under the fluorescent scope. A fluorescence of 2+ or greater was considered positive.

Adsorptions. Bacteria grown for 48 to 72 hr were washed 3 times with sterile saline. 0.1 ml of packed, washed bacteria was mixed with 0.5 ml of antiserum diluted 1:5 with saline. The mixture was incubated at 45-50 C for 2 hr and overnight at 4 C. Adsorptions were repeated until a negative reaction occurred when the adsorbed antiserum was reacted with the adsorbing antigen.

Microscopy. Microscopy was performed with an AO Spencer microscope, model 645, 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 10x occular lens and 45x objective lens.

Ouchterlony analysis. Antigens were prepared by washing bacteria four times with normal saline 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, New York) operating at 20 kH/s to produce at least 50% cell disruption as revealed by phase contrast microscopy.

C. vaginale cells required 30 min of sonication; the other corynebacteria required 5 to 10 min of sonication and L. acidophilus required 15 min of sonication. Sonication was performed with cell suspensions immersed in baths of acetone and dry ice. The 1/2 inch probe was cooled periodically. Sonicates were centrifuged for 15 min at 27,000 xg (Sorvall RC 2-B, Newtown, Conn.). The supernatants were lyophilized (Virtis Research Equipment, Gardiner, New York) and reconstituted to a concentration of 10 mg dry wt per ml distilled water. A negative control was prepared by removing the broth from an uninoculated flask and treating it as described above.

The diphasic broth was also utilized as a source of antigen for the Ouchterlony studies. The broth from a 48 to 72 hr culture was dialyzed against repeated changes of water at 4 C for three days. The dialysate was centrifuged at 27,000 xg for 20 min and the clear supernatant was lyophilized and reconstituted with five 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 1/4" x 4" glass slides and 5 ml of agar onto 1" x 3" glass slides producing an average of 1 to 1.5 mm

thickness. The agar had the following composition: 10 g/l Ionagar, (Colab, Chicago Heights, Illinois) 8.5 g/l sodium chloride (J. T. Baker Chemical Co., Phillipsburg, New Jersey) 1 g/l sodium azide, 37.5 g/l glycine (Matheson Co., Joliet, Illinois). Wells were cut in the agar so that the antiserum well measured 6 mm and the antigen well measured 3 mm. The peripheries of the wells were 5 mm apart. Reactions were read periodically and allowed to proceed for up to 72 hr at room temperature in a moist chamber. Slides were immersed in saline for 24 hr, followed by distilled water for 24 hr, evaluated again for precipitin lines and then removed from the water. To dry prior to staining the slides were covered with filter paper and allowed to air dry.

Staining was performed using Napthalene Black 12 B dye (Allied Chemical, Morristown, New Jersey) of the following composition: 1 g dye, 500 ml methanol (Mallinckrodt Chemical Works, St. Louis, Missouri) 200 ml glacial acetic acid (Mallinckrodt Chemical Works, St. Louis, Missouri) and 500 ml water. Slides were destained for 5 to 10 min in methanol: water: glacial acetic acid in a volume ratio of 7:2:1.

III. RESULTS

A. Biochemical characteristics. The C. vaginale strains tested 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. Non-hemolytic colonies measuring approximately 0.4 - 0.8 mm in diameter were visible in 36 to 48 hr and when stained appeared as gram variable diphtheroid-like organisms (Fig. 1A). 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 hr) 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 hr only masses of gram negative material were observed (Fig. 1B).

All the C. vaginale strains tested (Table 1) fermented glucose, maltose and starch, were non-hemolytic, 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

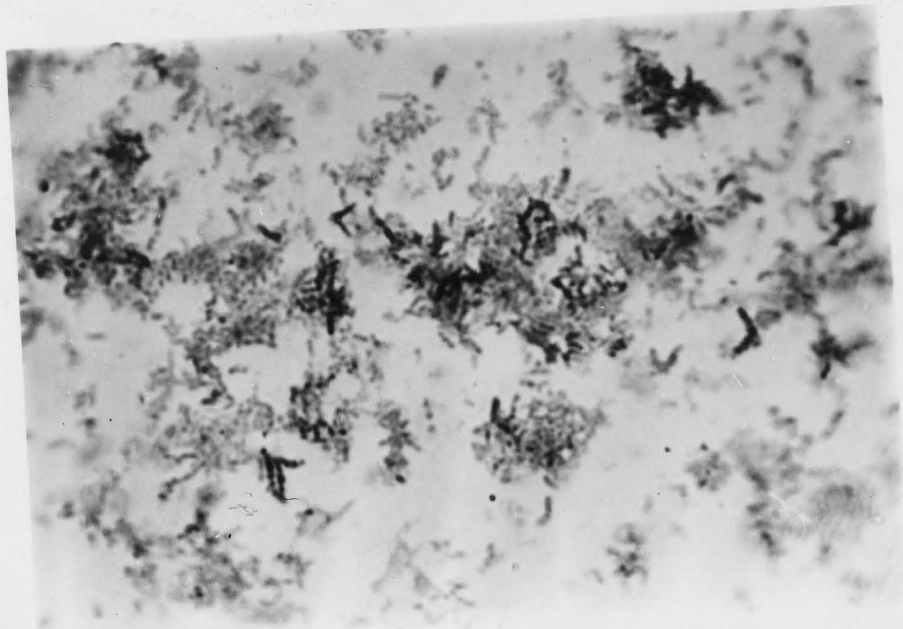

Fig. 1A. Gram stain of C. vaginale after 56 hr growth in PSD-thioglycollate diphasic medium. Magnification of this photograph is approximately 9,000.

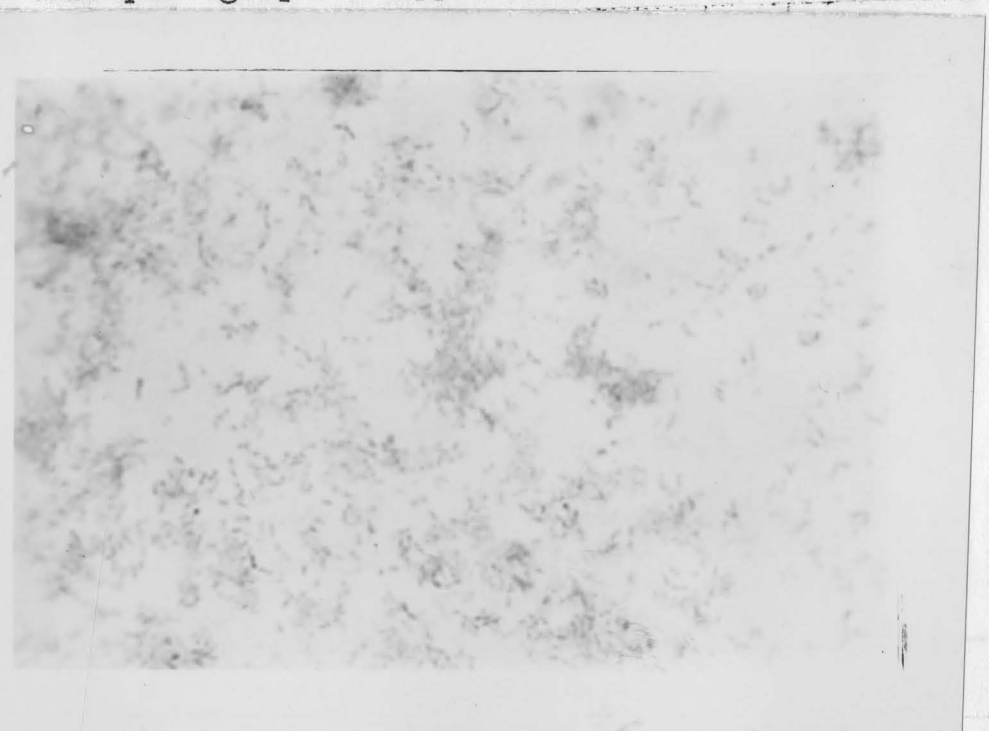

Fig. 1B. Gram stain of C. vaginale after 72 hr growth in PSD-thioglycollate diphasic medium. Magnification of this photograph is approximately 9,000.

TABLE 1

Biochemical Reactions of Various Corynebacterium sp.

Organism	PSD + Carbohydrate												
	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	A	-[b]	-	-	NH[c]	+[d]	-	-	-	+	-
594	A	A	A	-	-	-	NH	+	-	-	-	+	-
14018	A	A	A	A	-	-	NH	+	-	-	-	+	-
8226	A	A	A	A	-	-	NH	+	-	-	-	+	-
6488D	A	A	A	A	-	-	NH	+	-	-	-	+	-
6488W	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[e]
C.xerosis (7711)	A	A	-	-	-	-	NH	+	+	-	-	-	R
C.hofmanii(231)	-	-	-	-	-	-	NH	+	+	+	-	-	R
C.cervicis(13)	-	-	-	-	-	-	NH	+	+	-	-	-	R

[a]A=acid reaction, [b]-=negative reaction, [c]NH=no hemolysis, [d]+=positive reaction, [e]R=reduction.

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 non-hemolytic 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 positive methyl red test. C. xerosis strain 7711 fermented glucose and maltose; did not ferment starch, arabinose, xylose or mannitol; did not produce urease and produced a negative methyl red test. C. hofmanii strain 231 and C. cervicis 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.

B. Indirect fluorescent antibody studies.

1. Effect of fixation and culture medium upon fluorescence.

The effect of different methods of fixation 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.

2. Fluorescent staining reactions of C. vaginale 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-6488 D Di, and anti-6488 W Di) and one of the type strains grown on blood agar plates (anti-14018 Bld). The results obtained when employing the indirect fluorescent antibody staining technique are shown on Table 2. The antisera were reacted against six reference strains and ten clinical isolates which biochemically resemble C. vaginale. All reference strains fluoresced brightly following exposure to the homologous antiserum (Fig. 2A) but neither they nor the clinical isolates fluoresced following exposure to normal rabbit serum (Fig. 28). 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 fifteen of the sixteen organisms tested, and anti-8226 Di and anti-594 Di reacted with fourteen of the sixteen organisms tested.

The remaining antisera were less reactive. Anti-6488

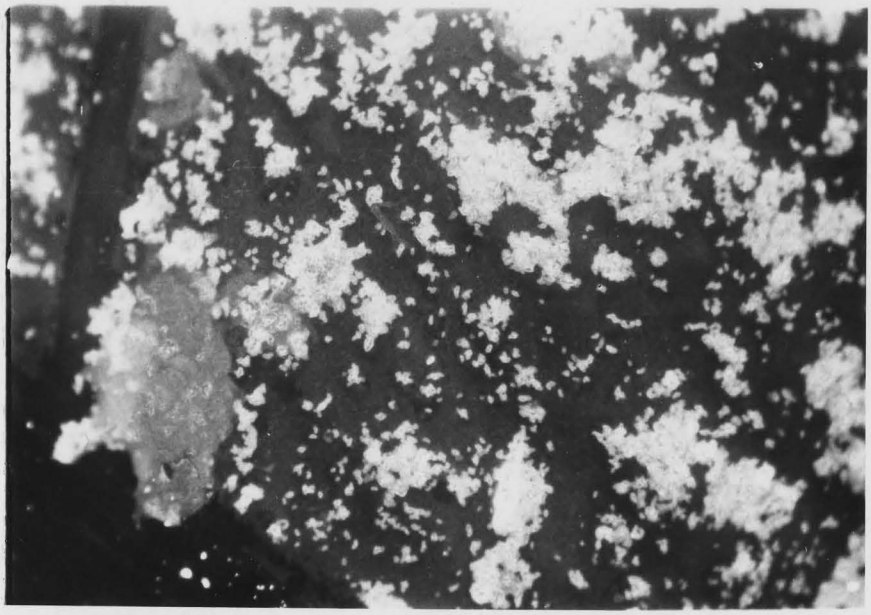

Fig. 2A. C. vaginale strain 14018 diphasically grown cells stained by the indirect fluorescent antibody technique with anti-14018. Magnification of these photographs is approximately x 4,5000.

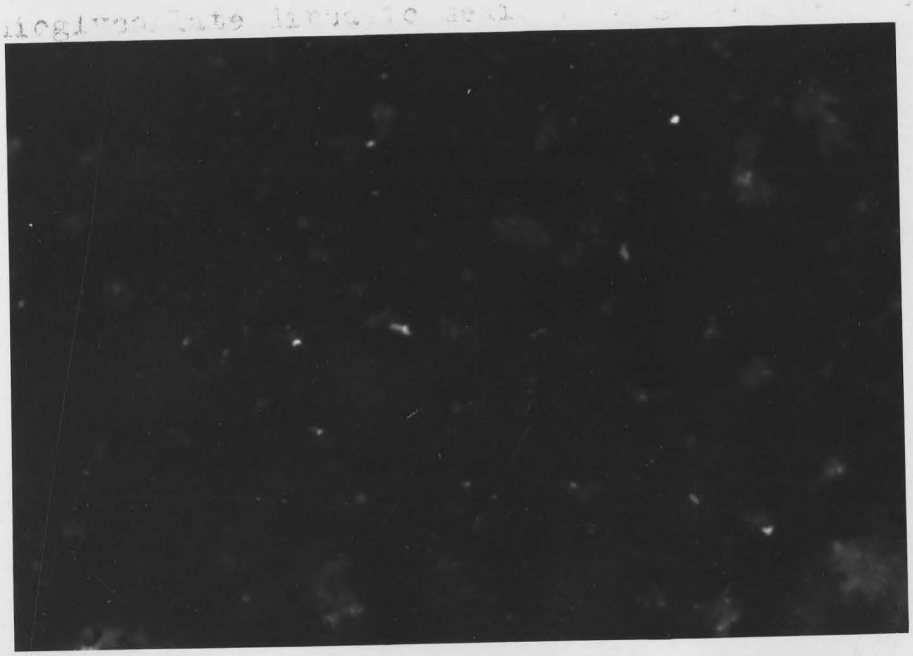

Fig. 2B. C. vaginale strain 14018 diphasically grown cells stained by the indirect fluorescent antibody technique with normal rabbit serum. Magnification of this photograph is approximately x 4,5000.

TABLE 2

Indirect fluorescent staining reactions of C. vaginale reference strains and clinical isolates with 1:10 dilutions of anti-C. vaginale.

Organism	Antisera							
	14018 Di[a]	8226 Di	594 Di	6488 Di	T94 Di	6488 W Di	14018 Bld[b]	NRS
Reference strains								
14018	3-4[c]	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] Di=organism grown diphasically

[b] Bld=organism grown on blood

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

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 also 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.

3. Fluorescent staining reactions of heterologous bacteria.

The specificities of the previous reactions obtained were assessed by reacting the seven antisera with possible related organisms chosen on the basis of morphology and site of infection and these reactions are shown in Table 3. All seven of the antisera reacted with L. acidophilus. Five of the antisera (anti-T94 Di, anti-594 Di, anti-8226 Di, anti-6488 D Di, and anti-6488 W Di) reacted with C. diphtheriae and two of the antisera (anti-14018 Bld and anti-8226 Di) reacted with C. xerosis. No fluorescence was obtained after staining C. cervices, C. hofmanii, H.influenzae, Actinomyces bovis, Nocardia asteroides, and Streptococcus 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 in the heterologous reactions, the titers of the cross-reactions

TABLE 3

Indirect fluorescent staining reactions of various bacterial strains and species with 1:10 dilutions of anti-*C. vaginale*.

Organism tested	Antiserum prepared against *C. vaginale* strain number (1/10)							
	T94 Di[a]	594 Di	14018Bld[b]	14018 Di	8226 Di	6488D Di	6488W Di	NRS
L.acidophilus (4356)	1-2[c]	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

Note: [a]Di=organism grown diphasically

[b]Bld=organism grown on blood agar plates

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

were determined. Using the criteria that a positive reaction requires at least a 2+ fluorescence, Table 4 indicates that all seven antisera gave titers of ten or under when reacted with C. diphtheriae. Six of the seven antisera gave titers of 10 or less when reacted with L. acidophilus. Anti-14018 Bld gave a titer of 80 when reacted against L. acidophilus and C. xerosis. Also, anti-8226 gave a titer of 80 when reacted against C. xerosis but none of the remaining antisera reacted with C. xerosis.

To further study the specificity of the heterologous fluorescence reactions, adsorptions were performed and the homologous titers compared before and after adsorption; antisera were adsorbed at least twice and a final adsorption was performed after a negative fluorescent reaction was obtained with the heterologous adsorbing organism; Table 5 shows the results obtained. There was no significant decrease in the titers of any of the seven antisera following adsorption with the apparent cross-reacting heterologous bacteria. Adsorbed antisera titers were decreased only one or two fold compared to the homologous strain titer. The homologous titers after adsorption with the cells of the cross-reacting bacteria were generally the same as that before adsorption and the fluorescence to the cross-reacting bacteria was eliminated.

4. Development of clinical method of identification of C. vaginale.

The previous results indicate that C. diphtheriae,

TABLE 4

Titers of *C. vaginale* antisera obtained with cross-reacting heterologous organisms detected by the indirect fluorescent antibody technique.

Antisera	Dilution					
	10	20	40	80	160	320
anti-*C. vaginale* vs. *C. diphtheriae* (11913)						
T94 Di[a]	1-2[c]	1-2	0-1	0-1	0-1	0-1
594 Di	1-2	0-1	0-1	0-1	0-1	0-1
8226 Di	2-3	1-2	1-2	0-1	0-1	0-1
6488 D Di	2	1	0-1	0-1	0-1	0-1
6488 W Di	1-2	1	0-1	0-1	0-1	0-1
anti *C. vaginale* vs. *L. acidophilus* (4356)						
T94 Di	1-2	0-1	0-1	0-1	0-1	0-1
594 Di	2-3	1-2	0-1	0-1	0-1	0-1
14018 Di	2	1-2	0-1	0-1	0-1	0-1
1226 Di	2-3	1-2	0-1	0-1	0-1	0-1
6488 D Di	1-2	0-1	0-1	0-1	0-1	0-1
6488 W Di	1-2	0-1	0-1	0-1	0-1	0-1
14018 Bld[b]	3-4	2-3	2	2	1	0-1
anti *C. vaginale* vs. *C. xerosis* (7711)						
14018 Bld	2-3	2-3	2-3	2	1	0-1
8226 Di	2-3	2-3	2	2	1-2	0-1

Note: [a]Di=organism grown diphasically.
[b]Bld=organism grown on blood agar plates.
[c]Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity.

TABLE 5

Homologous titers of C. vaginale antisera obtained before and after adsorption with heterologous cross reacting bacteria as detected by the indirect fluorescent antibody technique.

Antiserum	Adsorbing bacteria	Titer before adsorption	Titer after adsorption
anti T94 Di[a]	11913	2560[c]	1280
anti 594 Di	"	640	320
anti 14018 Di	"	320	160
anti 8226	"	320	320
anti 6488 D	"	640	160
anti 6488 W	"	80	40
anti 14018 Bld[b]	7711	640	320
anti 8226	7711	320	160
anti T94 Di	4356	2560	640
anti 594 Di	"	640	160
anti 14018 Di	"	320	320
anti 8226 Di	"	320	320
anti 6488 D Di	"	640	320
anti 6488 W Di	"	80	80
anti 14018 Bld	"	640	320

Note:
[a]Di=organism grown diphasically
[b]Bld=organism grown on blood agar plates
[c]Highest serum dilution resulting in at least a 2+ fluorescence.

C. xerosis and L. acidophilus when present in clinical specimens would be expected to fluoresce. Because they could not always be differentiated readily from C. vaginale on the basis of morphology, the antiserum 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. The three most highly reactive antisera were titered against C. vaginale reference strains and clinical isolates. Table 6 indicates that titers generally ranged from 80 to 320 for the reference strains. The titers for clinical isolates ranged from 20 to 640. However, generally the titers were between 20 to 40. When the antisera were subsequently tested at a 1/40 dilution, eight of the ten clinical isolates (8315, 1575, V28, V44, 359, 1637, 6234 and 1544) still gave a positive reaction with anti-594 Di, anti-14018 Di, or anti-8226 Di. At a 1/20 dilution significant fluorescent reactions were observed with all clinical isolates and reference strains but with certain clinical isolates the reactions were weak (2+) and borderline fluorescent reactions (1-2+) were observed with C. diphtheriae and L. acidophilus. Therefore, when diluted serum was used cross-reactions were reduced but not eliminated and it was thought that this might lead to confusion.

Adsorptions were performed in an attempt to eliminate nonspecific fluorescent reactions. Adsorbing anti-594 Di and anti-14018 Di with L. acidophilus also completely eliminated

TABLE 6

Titers of *C. vaginale* antisera with *C. vaginale* reference strains and clinical isolates detected by the indirect fluorescent antibody technique.

Organism	Titers of antisera anti 594 Di[a]	anti 14018 Di	anti 8226 Di
Reference strain			
T94	160[b]	160	80
594	640	640	320
8226	320	160	320
14018	320	320	160
6488 D	160	80	320
6488 W	80	160	80
Clinical isolates			
8315	640	160	160
1575	320	320	80
V28	40	40	80
V44	40	40	20
359	40	40	40
1637	40	40	20
1544	20	20	40
6234	40	20	20
144	0	20	0
8372	0	0	0

Note: [a]Di=organism grown diphasically
[b]Highest serum dilution resulting in at least a 2+ fluorescence.

their non-specific reaction with C. diphtheriae. However, anti-8226 Di adsorbed with L. acidophilus still gave a borderline fluorescent reaction (1-2+) with C. diphtheriae. Next, adsorbed anti-594 Di and adsorbed anti-14018 Di were reacted with the reference strains and clinical isolates to obtain an antiserum that would stain all strains of C. vaginale. Table 7 indicates that the adsorbed anti-594 Di reacted with all of the reference strains and seven of the ten clinical isolates tested. 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.

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.

C. Ouchterlony analysis of C. vaginale and possible related organisms.

1. Ouchterlony technique as applied to C. vaginale.

Preliminary immunodiffusion experiments were performed to determine the optimum conditions. C. vaginale strain 14018 cells were subjected to sonication for various times starting at 5 min and increasing at 5 min intervals to a maximum of 40 min. The lyophilized supernatants were reconstituted with distilled water and serial 2-fold dilutions were tested. The most distinct precipitin bands were produced utilizing the extract obtained after 30 min of sonication and

TABLE 7

Indirect fluorescent staining reactions of anti *C. vaginale* adsorbed with *L.acidophilus* versus *C. vaginale* reference strains and clinical isolates.

Organism	anti 14018 Di[a]	anti 594 Di	NRS[b]
Reference strain			
14018	3-4	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
C. diphtheriae	1	1	1

Note: [a]Di=organism grown diphasically
[b]NRS=normal rabbit serum
[c]Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity.

at a concentration of 1 mg dry wt/ml distilled water.

2. Effect of growth media upon antigen production.

In order to determine whether the type of growth media employed had an effect on the antigenicity of C. vaginale, extracts of sonicated cells of C. vaginale strain 14018 grown diphasically and on blood agar plates were reacted with antiserum prepared against this organism grown on both types of media. As shown in Fig. 3A extracts of diphasically grown cells (wells 2, 4 and 6) gave rise to two well defined precipitin bands when reacted with antiserum prepared against the organism grown on blood. In contrast, extracts of the blood grown cells (wells 1, 3 and 5) gave rise to a single precipitin band which coalesced with the "inner" band produced when the diphasically grown cells (wells 2, 4 and 6) were reacted with the antiserum prepared against the blood grown cells (Fig. 3A).

Figure 3B shows the reactions of the antiserum prepared against the same strain of C. vaginale grown diphasically (anti-14018 Di). Anti-14018 Di produced two precipitin bands with the extract of the blood grown cells (wells 1, 3 and 5) and with the extract of diphasically grown cells (wells 2, 4 and 6). A ring of identity formed early with the inner precipitin bands and is visible in Fig. 3B. Eventually the outer bands also coalesced indicating identity.

The broth extract (Fig. 4, 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

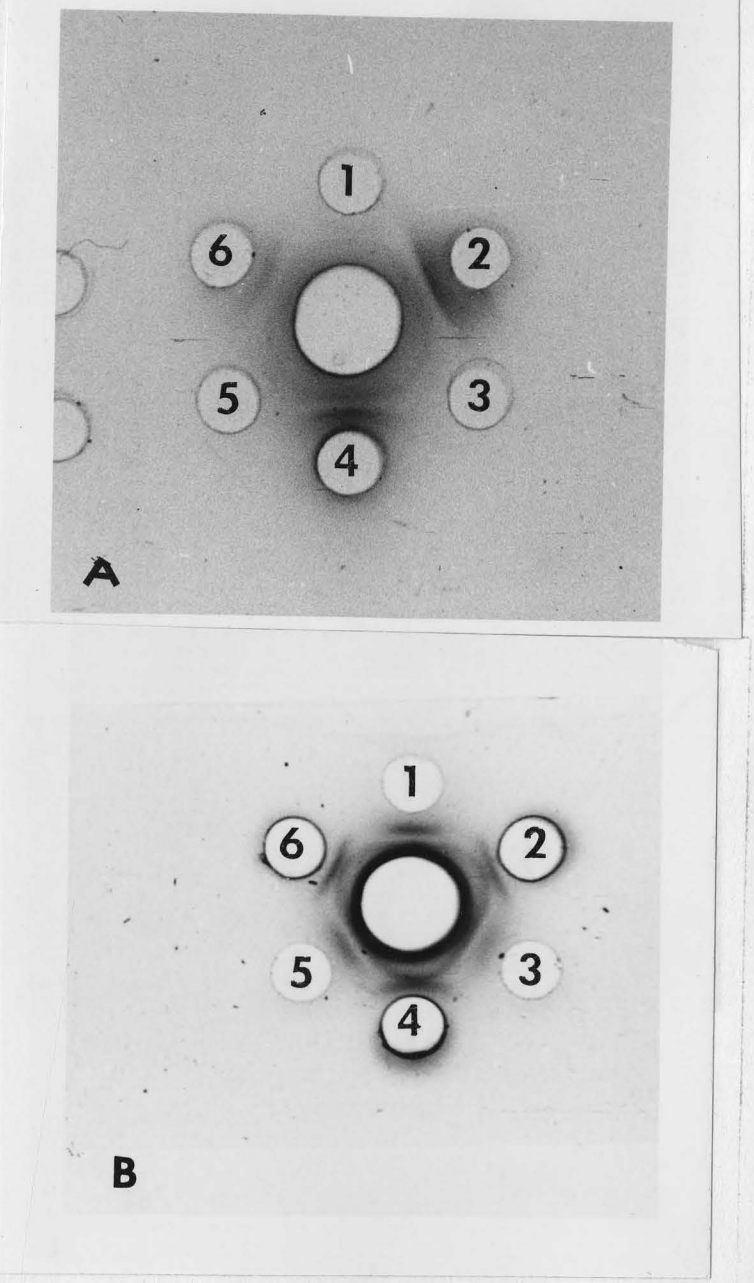

Fig. 3. Effect of growth media upon the production of C. vaginale strain 14018 antigens. Anti-14018 Bld (center well Fig. 3A) and anti-14018 Di (center well Fig. 3B) were reacted against the extract of sonicated 14018 blood grown cells (wells 1, 3 and 5) and the extract of sonicated 14018 diphasically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

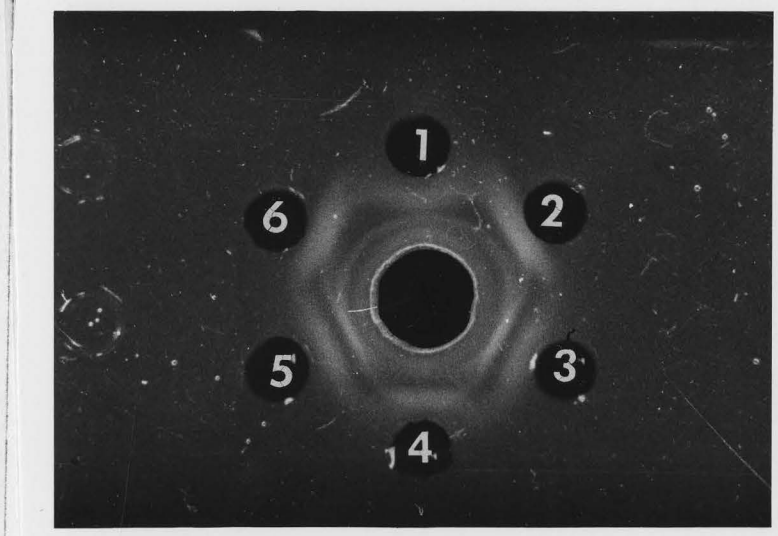

Fig. 4. Demonstration of diffusable antigens of C. vaginale strain 14018. Anti-14018 Di (center well) was reacted against the broth extract of strain 14018 (wells 1 and 4), the extract of sonicated blood grown cells (wells 2 and 6) and the extract of sonicated diphasically grown cells (wells 3 and 5).

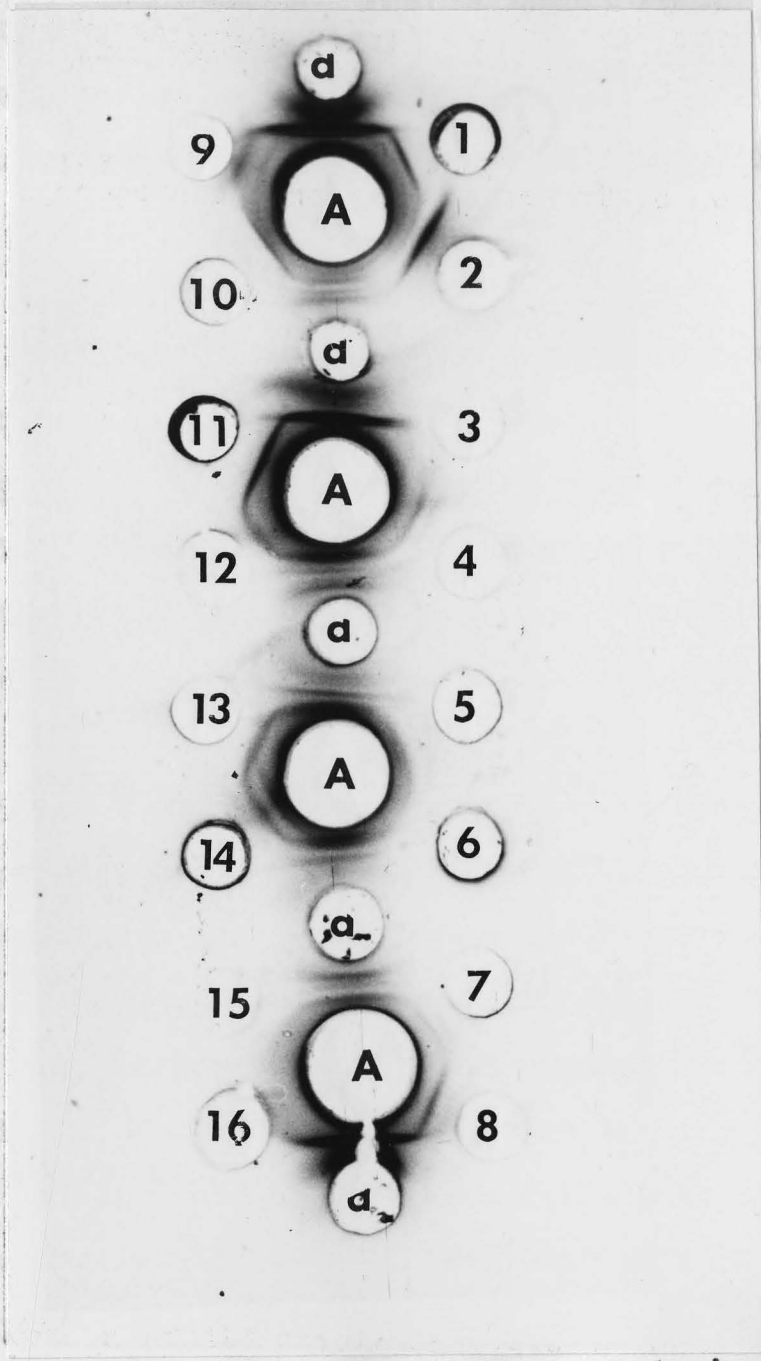

Fig. 5. Reactions between C. vaginale type strain 14018 and organisms which morphologically resembly C. vaginale. Center wells: A= antiserum prepared against C. vaginale grown diphasically, a= extract of 14018 sonicated cells grown. Outer wells: extracts of sonicated diphasically grown organisms which morphologically resemble C. vaginale (1) T94 (2) 594 (3) Negative control (4) 8236 (5) 6488 D (6) 6488 W (7) V28 (8) V44 (9) 144 (10) 359 (11) 1544 (12) 1575 (13) 1637 (14) 6234 (15) 8315 (16) 8372

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).

3. Cross-reactions.

Anti-14018 Di serum was subsequently employed in an attempt to demonstrate a common antigenic determinant among fourteen C. vaginale isolates. Although the number and intensity of bands varied, all C. vaginale isolates tested did share an antigenic determinant as indicated in Fig. 5. Bands of identity were visible with isolates 14018, 594, 8226, 6488 D, 6488 W, V28, V44, 359, 1544, 1575, 1637, 6234, and 8315 (wells a, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15 respectively). Spur formation indicative of partial identity appeared to be present with isolates T94 (well 1) and 144 (well 9). No bands formed with extracts of the vaginal diphtheroid 8372 (well 16) or the negative control (not shown). A curious feature of this system is that the extract of C. vaginale strain 14018 Di was found to produce four visibly distinct precipitin bands (Fig. 5, well a) with anti-14018 Di 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. 6, well a).

Possible related bacteria were included in this study

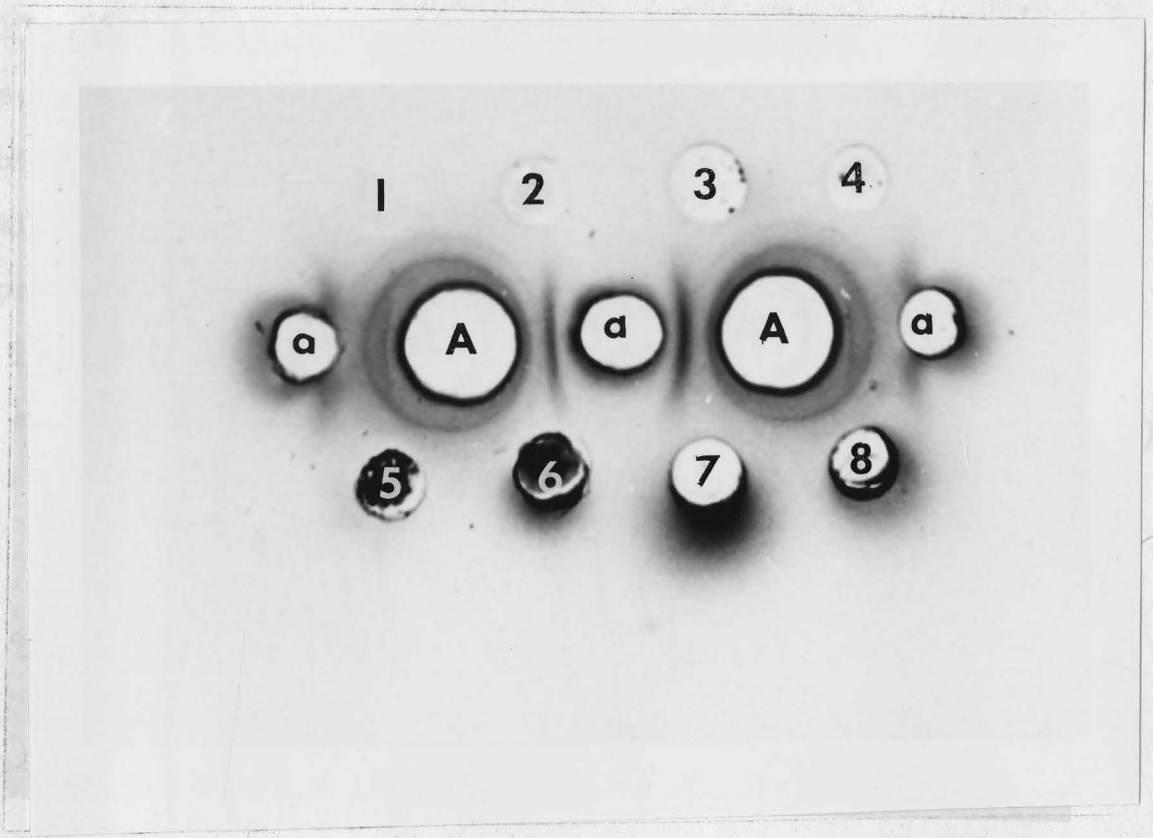

Fig. 6. Reactions between C. vaginale type strain 14018 and heterologous bacteria. Center wells: A= antiserum prepared against C. vaginale grown diphasically, a= extract of 14018 sonicated cells diphasically grown. Outer wells: extracts of heterologous bacteria (1) C. cervicis (2) C. xerosis (3) C. diphtheriae (4) L acidophilus (5) 318 (6) 6659 (7) Negative control

to determine the specificity of the Ouchterlony reactions. The bacteria were choosen 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 various vaginal diphtheroids (317, 6659) well 5 and 6 respectively, did not produce a precipitin band with anti 14018 Di (Fig. 6). Therefore, this Ouchterlony analysis did not detect the existence of a common antigenic determinant between C. vaginale and strains representative of the genera Corynebacteria or Lactobacillus. No reaction was observed with the control which consisted of an extract prepared from uninoculated diphasic medium (well 8).

IV. DISCUSSION

At present the identification of C. vaginale in clinical specimens is based on morphological and biochemical criteria. The method outlined by Drs. Dunkelberg and McVeigh (13) along with additional tests was employed in our laboratory to examine reference strains and isolates from clinical specimens which morphologically resembled C. vaginale.

All strains of C. vaginale used in this study were pleomorphic, varying from coccobacillary forms to diptheroid-like organisms with beading; were gram variable depending upon the age of the culture; were inhibited by H_2O_2; lacked catalase; and fermented glucose, maltose and starch. Arabinose was fermented by some strains whereas mannitol was fermented by none of the organisms. Xylose was fermented only by clinical isolate 8372 which was also the only isolate that produced a negative methyl red test. Dunkelberg (personal communication) noted that the colony morphology of 8372 was not typical of C. vaginale and this isolate did not ferment starch in his laboratory. This organism grew very slowly in the inoculation broth and it was difficult to get a sufficient number of organisms for the fermentation reactions. However under optimum conditions, this organism fermented all the carbohydrates tested except mannitol. Dunkelberg (personal communication) stated that 8372, "often failed to show typical C. vaginale form, being too small and lacking the central button." He

also noted, that it showed heavier growth on the PSD agar than is typical of most C. vaginale and had a yellowish tint rather than the white of typical C. vaginale isolates. The problems encountered with this isolate are indicative of the difficulties which arise when biochemical tests are used.

In general, the fermentation reaction pattern of C. vaginale is very difficult to determine. For example, the amount of growth in the inoculation broth is a critical factor. A broth with insufficient growth can lead to erroneously negative results. In contrast, an "overgrown" broth may contain (a) many dead or dying organisms which may cause slow fermentation reactions or erroneously negative results, and/or (b) a high concentration of acid which may cause an immediate color change in the medium upon addition of the initial inoculum. Therefore, a negative control tube containing the basic medium but lacking carbohydrate is essential. Secondly, contamination can readily occur because the medium is designed to support fastidious organisms and many transfers are essential. Contamination was checked by: (a) subculturing and gram staining the inoculation broth, (b) noting the time of fermentation; that is, if very rapid fermentation occurred, the fermentation tube itself was checked for contamination, and (c) confirming the results by repeated testing. Consequently, it can be seen that biochemical tests are time consuming, cumbersome, and are subject to error.

Hence, 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. Problems of autoagglutination are frequently encountered 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. Redmond and Kotcher (33) reported that dispersing the organism in Hank's balanced salt solution and bovine albumin eliminated this problem. When we employed this technique the isolates which we examined still autoagglutinated.

However, fluorescent microscopy can overcome both of these difficulties. It was noted that the fluorescent technique is specific, sensitive, rapid, simple, economical and becoming widely used in the clinical laboratory. Therefore the establishment of a serological method of identification employing the fluorescent antibody technique was attempted.

Antisera were prepared in rabbits against the six reference strains and were tested against homologous and heterologous organisms by the indirect fluorescent antibody technique. Differences in reactivity were noted among the antisera. Three of the antisera were highly reactive and produced a positive reaction with all the type strains. The remaining four antisera varied in reactivity against the type strains: anti-6488 W Di reacted only with the homologous strain; anti-14018 Bld reacted with all the reference strains except 6488 W; and

anti-T94 Di and anti-6488 D Di reacted with 14018 and 6488 D but varied in reactivity to 594, T94, 8226 and 6488 W. These variations may be accounted for by: (a) differences in amount of antigen present, (b) lack of a specific antigen in some strains, (c) mutations during transfer, or (d) differences in antibody response of the rabbits. Previous investigators have observed that the immunological response of various rabbits to antigen may be quite different (31).

Differences were also noted in antisera prepared against the "same" strain obtained from two different laboratories. For example, subtle differences were observed between anti-594 and anti-14018 Di, but very striking differences were observed with anti-6488 D and anti-6488 W. Antiserum prepared against 6488 W,which was obtained from Dr. Weaver,reacted only with two or the eight clinical isolates tested and none of the reference strains. In contrast, antiserum prepared against 6488 D, supposedly the same strain but obtained from Dr. Dunkelberg, reacted with nine of the fifteen isolates tested. These variations may be accounted for by: (a) a change in the antigenic structure of the organism brought about by the method of transferring and/or the medium employed and/or (b) non-identity of the strains examined, and/or (c) differences in the antibody response of the rabbits.

It was noted that unusual non-reciprocal 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 (9) observed a similar nonreciprocal reaction between strains of Rhizobium japonium. He concluded that either (a) cross-reacting antibodies were involved or (b) the antigenic determinant is present but in a limited amount.

Several reports (29, 34) 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 choosen 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 Streptococcus failed to fluoresce with each C. vaginale antiserum. Cross-reactions did occur with C. diptheriae, C. xerosis, and L. acidophilus. Generally, titers of ten 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 absorbing the antisera with the heterologous bacteria,

homologous titers were decreased only one or two fold.

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 immunological method for identification of C. vaginale various techniques were utilized in an attempt to eliminate the non specific 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. Previous reports in the literature have noted a similar problem (29, 34).

However, it was possible to selectively remove the responsible cross-reacting factors by adsorption. Each of the three highly reactive antisera cross-reacted with L. acidophilus and two of these antisera also reacted with C. diptheriae. Subsequently these antisera were adsorbed with L. acidophilus and when tested for reactions with C. diptheriae and L. acidophilus two antisera (anti-594 Di and anti-14018) exhibited negative fluorescence. Adsorbed antiserum retained its ability to stain homologous organisms brilliantly whereas heterologous antigens exhibited only a 0 to 1+ fluorescence.

When the adsorbed antiserum was tested against the reference strains and clinical isolates biochemically resembling C. vaginale, adsorbed anti-14018 gave a definite positive reaction with all C. vaginale organisms tested.

The specificity of the reaction was further tested by adsorbing anti-14018 with the homologous strain. When this adsorbed antiserum was reacted with the C. vaginale reference strains and clinical isolates, no fluorescence was observed.

The type of culture medium on which the organism is grown may also play an important role in its serological reactions. Antisera 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 media employed (29).

Additional evidence that the type of medium employed plays a major role in the type of antiserum prepared has been obtained by Ouchterlony analysis. 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. These results may be due to the fact that the (a) diphasic media stimulates an increased production of a specific antigenic determinant present on C. vaginale cells and/or (b) when the cells are grown in the diphasic media the antigen is more readily available for antigenic recognition.

Studies were performed utilizing the diphasic broth in which C. vaginale organisms had been grown in order to determine whether diffusable antigens were produced. The extract of the broth in which 14018 was grown (diphasically) gave a single precipitin band which coalesced with one of the bands produced both by cell extracts of 14018 grown on blood agar plates and with cell extracts of 14018 grown diphasically. Thus, it appears that C. vaginale produces at least one antigen of high molecular weight which is released relatively easily from the cells into the medium.

Fifteen isolates of C. vaginale were shown by the Ouchterlony technique to share a common antigenic determinant. This provided additional support to our earlier characterization of these isolates made on the basis of biochemical reactions and fluorescent microscopy. A reaction of identity was observed between the type strain 14018 and twelve of the C. vaginale isolates. Bands of partial identity appeared to form with two isolates and the type strain; however, it is believed that these 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. Antigen excess was considered as a possible cause for the coalescence of bands;

however, diluting the extract did not increase the number of precipitin bands. Furthermore, the concentration of the homologous reactants was the same in all systems tested. The area between wells was increased to 7 mm in an attempt to spread the bands apart; however, again only two bands were distinguishable with the homologous system. The reason for the enhancement by the related bacteria is obscure at present. However, previous reports possibly lend an insight into this phenomenon. Previous investigators (8, 19) have noted that heterologous reactions may give rise to a greater number of bands. Also reports have been published (23) demonstrating that the sensitivity of immunodiffusion tests is inhanced by bordering a well containing a weak reactant unable to produce a visible precipitate with a well containing a greater concentration of this reactant. Possibly the heterologous strain possesses a different concentration of a specific antigenic determinant than does the homologous strain which (a) tends to enhance the formation of a band previously not visible or (b) 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.

We were unable to detect an antigenic relationship between C. vaginale and members of the genera Corynebacterium or Lactobacillus employing either the indirect fluorescent or Ouchterlony techniques. Nor were there any relationships detected between C. vaginale and vaginal diptheroids which

morphologically could be confused with C. vaginale.

Therefore the evaluation studies in this laboratory with reference strains and clinical isolates demonstrated that fluorescent microscopy is as specific and sensitive as the conventional biochemical tests, and is also a more rapid method of identification. Also, it appears that the Ouchterlony technique, although time consuming, could be employed as a specific method for detecting C. vaginale. However, the pitfalls of sonication such as overheating 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 which will answer the problems associated with the proper identification and taxonomic classification of this organism.

V. SUMMARY

The indirect fluorescence and Ouchterlony techniques were employed to study the antigenic structure of C. vaginale and to develop a rapid method for the identification of this organism. Antisera prepared in rabbits against six reference strains of C. vaginale were used to compare the reference strains and ten clinical isolates which were selected on the basis of morphology and conventional biochemical tests.

When the indirect fluorescent antibody technique was utilized, certain of the antisera did exhibit a cross reacting titer when reacted against Corynebacterium diphtheriae, Corynebacterium xerosis, or Lactobacillus acidophilus. However, antisera adsorbed with the heterologous bacteria did not exhibit a significant decrease in titer when reacted against the homologous strain. Various other species of corynebacteria 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 Lactobacillus acidophilus. The adsorption removed the cross-reacting antibody but did not affect the staining reaction with Corynebacterium vaginale strains. All reference strains and clinical isolates characterized as Corynebacterium vaginale gave a definite positive reaction with the adsorbed anti-14018 Di. The specificity of the reactions was assessed by adsorbing the antisera with the homologous strain. The data suggest that

the indirect staining method will be of value in the rapid identification of Corynebacterium vaginale.

The immunodiffusion technique was also employed to study the antigenic relationship of the fifteen C. vaginale isolates, several vaginal diptheroids, and members of the genera Corynebacterium and Lactobacillus. Antisera prepared against the ATCC strain 14018 grown diphasically and on blood agar plates, were tested against extracts of the organism prepared by sonication. Ouchterlony analysis demonstrated that all fifteen 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.

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APPROVAL SHEET

The thesis submitted by Mary Frances Smaron has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

1/8/73
Date

John L. Vice

Advisory Committee:

1. Dr. John L. Vice
2. Dr. T. Hashimoto
3. Dr. Leon Marcus