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Comparison of Specimen Collection and Laboratory Techniques for Isolation of *Haemophilus ducreyi*

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Sixteen patients with clinical chancroid were studied prospectively; different culture media and sampling techniques from genital lesions were evaluated. Technique A was aspiration of a saline wash from the ulcer, which was pooled and inoculated into rabbit blood, rabbit blood + vancomycin (5 μg/ml), and semisolid chocolate agar + vancomycin (3 μg/ml). Each primary culture medium was subcultured to chocolate agar with 1% IsoVitaleX (CA), CA with vancomycin (3 μg/ml; CA + v), and CA with vancomycin (3 μg/ml) plus polymyxin (7.5 μg/ml; CA + vp). Technique B was the use of a cotton swab, plated directly on CA, CA + v, and CA + vp. Nine strains of *Haemophilus ducreyi* were obtained. Technique A yielded seven strains, whereas technique B yielded eight strains; with each technique, five strains were isolated only after use of selective antibiotic media. CA + v medium yielded the largest number of isolates. Direct inoculation by swab to CA + v from chancroidal ulcers is effective as an isolation technique for growth of *H. ducreyi*.

Chancroid, a rare venereal disease, is usually diagnosed by a clinician (3). No useful serological diagnostic techniques are available, and the definitive diagnosis of chancroid depends upon isolation of the etiological agent, *Haemophilus ducreyi*. However, obtaining a pure culture of this organism from the genital sore of chancroid patients has usually not been possible, with isolation rates varying from 0 (8, 11) to 9 (14) to 25% (18). Deacon obtained a 75% isolation rate in 20 patients utilizing fresh rabbit blood (RB) used within 2 h of collection, an impractical technique for most laboratories (4). Although the clinical impression has often been confirmed in the past by Gram stain of genital pus (1, 6), by the chaining morphology of the organism in liquid media prepared with RB or human blood (3, 17, 18), by biopsy (7), by therapeutic trial, and by use of the delayed cutaneous reaction to an antigen prepared from *H. ducreyi* (5, 16), each of these techniques has serious disadvantages. The Gram stain of ulcer pus is relatively nonspecific, as other bacteria, particularly *Bacteroides* species, are often present in these lesions and give a similar appearance on the Gram stain (4). The use of complement-inactivated RB frequently results in failure to isolate the organism due to overgrowth by contaminants (4); biopsies of these genital lesions are usually not done because they are very painful; and commercial *H. ducreyi* antigen is no longer available for the skin test.

In a recent outbreak of chancroid in Winnipeg, Canada, using standard culture techniques, a low yield of *H. ducreyi* was obtained after primary inoculation into clotted RB and subculture to chocolate agar with 1% IsoVitaleX (CA). Only five genital lesions from 32 patients yielded this organism. In an additional six patients typical chaining gram-negative organisms could be visualized in RB but could not be isolated. We found a high isolation rate of gram-positive contaminant organisms, especially diphtheroids, *Staphylococcus epidermidis*, and streptococci but very rarely *Enterobacteriaceae*. A screening survey with three of our initial isolates of *H. ducreyi* to several antibiotics, using a disk susceptibility technique, showed that these isolates were relatively resistant to vancomycin and polymyxin individually. We then initiated the present evaluation of selective media for isolation of this organism from patients with chancroid lesions, using chocolate agar (CA) in which vancomycin alone (CA + v) or vancomycin plus polymyxin (CA + vp) was incorporated to eliminate gram-positive and gram-negative contaminants. This isolation rate was compared to the standard technique that uses RB.

MATERIALS AND METHODS

Beginning in December 1976 specimens were collected from genital ulcers by two sampling techniques from all patients in whom chancroid was suspected in the Ambulatory Care facilities at the Health Sciences

Centre, Winnipeg, Canada. Eighteen patients were studied, and all had three negative dark-field examinations. Before a sample was taken, the ulcer base was throughly cleaned with a gauze pad moistened in normal saline. One patient had a contaminated draining sinus from his groin bubo at the time of presentation, which for purposes of this study was treated as a genital ulcer.

Technique A. Technique A was always performed first. It consisted of irrigation and agitation of the genital ulcer base, using 0.5 ml of non-bacteriostatic saline in small portions and aspiration with a smooth-tipped Pasteur pipette. This specimen was mixed by vortex agitation for 30 s and 3 to 4 drops was placed in the three primary liquid culture media. The first two liquid media each contained 3 ml of clotted RB, collected up to 1 month previously and stored at 4°C. Vancomycin in a concentration of 5 μg/ml was added to the second tube. The third liquid medium contained 3 ml of semisolid CA + v in a concentration of 3 μg/ml. This concentration of vancomycin has been shown to inhibit over 90% *S. epidermidis* and *S. aureus* in vitro (15). After 24 to 48 h of incubation, each primary liquid culture medium was Gram stained and subcultured on three secondary media: (i) a CA plate composed of gonococcal medium base (Difco), enriched with 1% hemoglobin (Difco) and 1% IsoVitaleX (BBL); (ii) a CA with v, 3 μg/ml; and (iii) CA with v, 3 μg/ml, and polymyxin B (Sigma), 7.5 μg/ml. This is depicted by the flow chart of Fig. 1. When nonchaining gram-negative rods were observed in the primary RB culture, subculture to sheep blood agar with anaerobic incubation was performed.

Technique B. Technique B consisted of sampling the cleaned ulcer base with a cotton swab moistened with saline, which was then immediately streaked on the surface of a CA plate, followed by streaking on a CA + v plate and then a CA + vp plate containing the antibiotic concentrations mentioned previously (Fig. 1). All cultures were incubated at 33°C in 5 to 10% CO_2 in the presence of a saturated water vapor atmosphere in a Hotpack CO_2 incubator. Swabs of genital ulcers were taken from five patients for herpesvirus culture and placed in Hanks balanced salt solution for transport to the Virology Laboratory of the Cadham Provincial Laboratory.

In an attempt to define their taxonomic position in the *Haemophilus* family, biochemical tests as described by Kilian (9) were carried out on all our strains (19 to date) as well as 4 reference strains (CIP A75, A76, A77, and 54.2 from l'Institut Pasteur, Paris, France). These tests included examination for porphyrin production from δ-aminolaevulinic acid, V-factor requirement, production of indole and H_2S, nitrate reduction, enzymatic action of urease, ornithine decarboxylase, oxidase, catalase, alkaline phosphatase, and fermentation of glucose, sucrose, lactose, xylose, mannitol, and deoxyribose. Controls for these biochemical tests included one strain of *Haemophilus influenzae* biotype 1, one strain of *H. parainfluenzae* biotype 1, and one reference strain of *H. paraphrophilus* ATCC 29242.

RESULTS

Organisms considered to be *H. ducreyi* showed the following characteristics. Nonmucoid yellow-grey translucent colonies that could be pushed virtually intact across the agar surface appeared from 2 to 9 days after inoculation (4, 18). On Gram stain the organisms were seen in pleomorphic clumps and short chains when examined from the agar surface and exhibited longer chains when grown from a liquid medium. The isolates obtained from this study had the same appearance as 10 of our previous strains, 4 of which were obtained in pure culture from patients with fluctuant inguinal buboes. We have used the term strain to refer to the organisms found from each different patient.

Results of the biochemical tests as studied by Kilian for *Haemophilus* species conformed with Kilian's in all respects, except that three reference and one Winnipeg isolate of *H. ducreyi* did not reduce nitrate.

Anaerobic cultures performed where nonchaining gram-negative rods were visualized on Gram stain of the RB culture yielded *Bacteroides melaninogenicus* in three cases with and one without concomitant *H. ducreyi* isolation. In two patients *H. ducreyi* was isolated with no *Bacteroides* species present. A few colonies of aerobic gram-negative organisms were cultured in five cases, but contamination by these organisms was negligible.

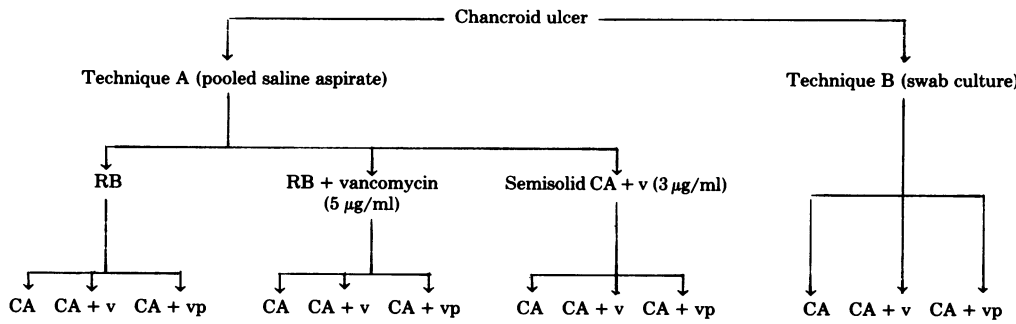
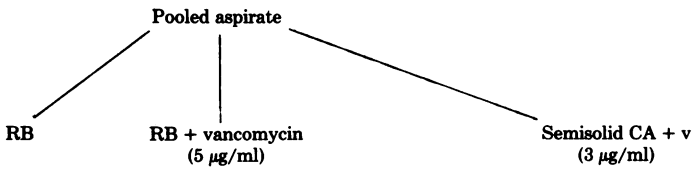


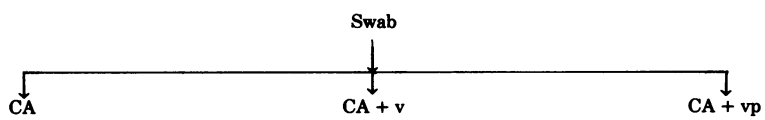
FIG. 1. *Protocol for isolation of H. ducreyi.*

The results are summarized in Fig. 2 and 3. Two patients were dropped from the study, one because of a positive culture of herpes virus, and one grew pure *S. aureus* with no gram-negative organisms seen at initial Gram stain or on culture; this patient also had a history of genital trauma and no typical contact history for chancroid. Nine strains of *H. ducreyi* were recovered from the remaining 16 patients. Four of the culture-negative cases deserve special comment. One female patient had the typical chaining morphology observed in RB medium, but no *H. ducreyi* could be isolated due to overgrowth of *Neisseria gonorrhoeae,* which also has a selective advantage on these media due to its resistance to vancomycin and poly-



	Strain		Strain		Strain	
CA subculture	54202	2 colonies after 3 days	Nil		54202	4 colonies after 3 days
	54209	2 colonies after 3 days			54209	2 colonies after 3 days
CA + v subculture	54198	3 colonies after 9 days	54213	4 colonies after 6 days	54211	2 colonies after 4 days
	54204	15 colonies after 6 days			54213	2 colonies after 6 days
CA + vp subculture	54204	2 colonies after 5 days	Nil		Nil	
	54205	1 colony after 6 days				

FIG. 2. *Isolates from pooled aspirate—technique A.*



Strain		Strain		Strain	
54198	2 colonies after 3 days	54201	1 colony after 4 days	54204	1 colony after 5 days
54201	2 colonies after 8 days	54202	2 colonies after 3 days		
54202	2 colonies after 4 days	54204	>25 colonies after 2 days		
		54207	4 colonies after 3 days		
		54209	>20 colonies after 3 days		
		54211	8 colonies after 4 days		
		54213	1 colony after 3 days		

FIG. 3. *Isolates from swab sampling—technique B.*

myxin. The lesions in two male patients with negative cultures were only 1 to 2 mm in diameter and nonpurulent, suggesting very early chancroid. A fourth patient had been receiving oral cloxacillin at the time the culture was taken. Seven strains were cultured by more than one technique; e.g., 54202 and 54204 were cultured by four different methods. Eight different strains were recovered with the direct swabbing technique, whereas seven were recovered from pooled secretions. Of the eight strains recovered from direct swabbing on agar, seven were recovered from the CA + v, whereas only three were recovered from the surface of CA alone and only one from CA + vp. The number of colonies on the surface of these media differed considerably, with only 1 to 2 colonies visible on CA, whereas four of seven isolates on CA + v had 4 to 25 colonies on the plate. The growth of these colonies on agar was very slow, being observed from 2 to 9 days after inoculation, with a medium of 4 days required to achieve sufficient growth for recognition. Most of the contaminating organisms were gram positive as shown by the marked reduction in extraneous flora in CA + v plates.

DISCUSSION

We believe that the clinical picture of chancroid can be supported by laboratory isolation of *H. ducreyi* from genital ulcers in a large number of cases. Although uncertainty exists about the identification and association of this organism with chancroid, our isolates conform closely to the CIP strains examined by Kilian and lend support to his suggestion that the classification of *H. ducreyi* be restricted to hemin-dependent gram-negative streptobacillary organisms (9). In the present series, isolates of this organism in pure culture were obtained from 56% of a group of patients with probable chancroid lesions. There was little difference in isolation rate from the two sampling methods, with seven and eight strains isolated by the aspiration and swab technique, respectively. Possible reasons for failure to isolate these organisms in the remaining 44% included early lesions, concomitant *N. gonorrhoeae* infection, and concurrent antibiotic treatment. Certainly, familiarity with the colonial appearance of the organism contributed to the overall yield, and incubation conditions previously described should be closely followed for growth of this fastidious bacterium.

The usefulness of selective media has been shown to be important for isolation of the gonococcus (12, 19), *H. influenzae* from the pharynx (13), and group B streptococci from genital flora (2). Selective media presumably allow for easier detection of the slower-growing pathogens by suppression of faster-growing contaminants and elimination or reduction of their toxic inhibition or consumption of nutrients (10). In this study isolation on CA was improved by the inclusion of vancomycin (CA + v), but CA + vp, in the concentration employed, was inhibitory and yielded a lower isolation rate than the nonselective CA alone. Thus, a simple isolation technique for *H. ducreyi* is a cotton swab moistened in saline to sample the cleaned ulcer base followed by streaking on the surface of CA and to a selective medium of CA + v, 3 μg/ml. In our series, this simple sampling and cultural technique yielded 8 of the 9 different strains from 16 patients; seven of these eight strains were isolated on the vancomycin-containing plate. Also, with contamination reduced on the vancomycin plate, the number of colonies of *H. ducreyi* on the selective medium was increased and the work simplified. In comparison, the traditional RB medium eventually yielded six strains overall, but four were only isolated on subculture to selective antibiotic-containing media.

Enriched CA is as an effective primary culture medium in place of RB. Improvements in sampling techniques and a greater understanding of the nutritional requirements of this organism may further increase its isolation rate.

Confirmation of chancroid by isolation of *H. ducreyi* is useful for patient management, in the epidemiology of veneral disease, and for further laboratory studies on this interesting organism.

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