

Isolation and identification of *Haemophilus ducreyi* in a clinical laboratory

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Summary. Routine procedures used to isolate *Haemophilus ducreyi* in a busy laboratory are reported. Identification was based on colony morphology and nutritional and biochemical properties of 120 fresh isolates of *H. ducreyi*. These isolates grew very well on Gonococcal Agar and Mueller-Hinton Agar incubated at 34°C in candle extinction jars containing moistened filter paper. Colonies varied in size, giving a polymorphic appearance. They were smooth, dome-shaped, and buff-yellow to grey in colour, and measured 2 mm in diameter. They could be pushed intact across the agar surface. By microscopic examination of gram-stained smears the isolates were gram-negative coccobacilli arranged in short chains, clumps or whorls and occasionally in typical “rail track” arrangements. Individual bacteria showed bipolar staining. Colonies autoagglutinated in saline. All strains were catalase-negative and did not produce indole or H₂S. They were oxidase- and β -lactamase positive and required X but not V factor for growth. Now that reliable techniques have been developed and characteristics established it is possible for most clinical laboratories to isolate and identify this organism from most patients with chancroid.

Introduction

Chancroid is a common sexually-transmitted disease (STD) in many parts of the tropics (Kibukamusoke 1965; Nsanze *et al.*, 1981). Evidence of sporadic outbreaks in various temperate countries over the past few years also exists. The causative organism, *Haemophilus ducreyi*, was recognised in stained smears of ulcer exudates by Auguste Ducrey in 1889, but his attempts to culture it were unsuccessful. The first isolation has been variously credited to Lenglet in 1898, Bezancom, Griffin and LeSourd in 1900, Petesen in 1895 or Istoamanov and Akopiantz in 1897 (Ronald and Albritton, 1984); these early workers used media and techniques of unknown sensitivity and specificity. Until recently, examination of gram-stained exudate was relied upon for the laboratory diagnosis of the soft chancre, despite its dubious reliability (Chapel *et al.*, 1977; Nsanze *et al.*, 1981; Choudhary *et al.*, 1982). Because of difficulties encountered in isolating the organism by culture and the gradual disappearance of endemic chancroid in industrialised nations, interest in the bacterium waned.

A resurgence of interest occurred when Hammond *et al.* (1978a) developed a selective agar for the isolation of *H. ducreyi* during an urban epidemic of chancroid in Canada. Their medium consisted of Gonococcal Agar with bovine haemoglobin 1%, Isovitalex 1% and vancomycin 3 mg/L. Nsanze *et al.* (1981) reported the isolation of *H. ducreyi* from 70% of patients with clinical chancroid in Nairobi using Hammond's medium modified by the addition of 5% fetal calf serum. Similar isolation rates of *H. ducreyi* were obtained in Johannesburg by Bilgeri *et al.* (1982); they used Mueller-Hinton Agar with chocolate horse blood 5%, Isovitalex 1% and vancomycin 3 mg/L. Nsanze *et al.* (1984) compared Gonococcal Agar medium with Mueller-Hinton Agar medium for the primary isolation of *H. ducreyi* from genital ulcers in Kenya and found that the use of the two media together increased the yield of positive cultures; they recommended the use of both in STD laboratories.

H. ducreyi has few demonstrable biochemical characteristics (Ronald and Albritton 1984) because of its fastidious growth requirements. Reports on the key identification tests of this organism grown on different media are conflicting (Sottnek *et al.*, 1980; Sturm and Zanen, 1984; Taylor *et al.*, 1984). Furthermore, there is speculation that different biotypes of this organism may

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exist in different parts of the world (Sturm and Zanen, 1984). This situation may be due to lack of well-defined techniques for growing the organism on proven media leading to specific identification. We now report the development of routine isolation and identification procedures and observations on the morphological, nutritional and biochemical characteristics of *H. ducreyi* strain isolated on the two media in Nairobi during the period Jun.–Sep. 1984.

Materials and methods

Patients and specimens

Male patients with genital ulcers were examined on presentation at the Nairobi City Special Treatment Clinic (STC). If any ulcer had a dry scab this was elevated and removed with the broken end of a swab stick. Dry cotton swabs were used to collect exudates from the bases of the lesions. In the clinic, specimens were inoculated directly (but not spread) on two media: GC Agar base (Gibco Diagnostic, Madison, WI) containing bovine haemoglobin 2%, Isovitalex 1% and vancomycin 3 mg/L (GC-Hgs) Mueller-Hinton Agar base (Baltimore Biological Laboratories, Becton-Dickinson Company, Cockeysville, MD) containing chocolate horse blood 5%, Isovitalex 1% and vancomycin 3 mg/L (MH-HB).

Isolation and identification of strains

On return to the laboratory 2–3 h later, the GC-Hgs and MH-HB plates were streaked with wire loops to give single colony isolations. The plates were then incubated at 34°C in a candle extinction jar with a moistened filter paper to provide a CO₂-enriched, water-vapour saturated atmosphere. Plates were examined after incubation for 48 h and those which showed poor growth or none were reincubated and re-examined daily for up to 5 days. *H. ducreyi* isolates were identified by typical colony morphology and colour, and appearance in gram-stained smears.

Biochemical tests

Catalase activity was measured by dropping H₂O₂ 5% v/v on the colonies on clean glass slides. *Staphylococcus aureus* and *Streptococcus pyogenes* were used as control organisms.

Oxidase production was detected by the method of Kovacs (1956) with tetramethyl-p-phenylenediamine; *Escherichia coli* and *Neisseria gonorrhoeae* were control organisms.

β-Lactamase activity was detected with chromogenic cephalosporin (Nitrocefin, Glaxo) in a cup of a microtitration plate, with *S. aureus* and *Str. pyogenes* as control organisms.

H₂S production on Isovitalex-enriched chocolate agar medium was sought with lead-acetate paper (Cowan 1974). *Proteus vulgaris* and *E. coli* were used as control organisms.

Indole production was detected by the method of Clarke and Cowan (1952) with *E. coli* and *Klebsiella pneumoniae* as controls. Furthermore X- and V-factor tests were performed with impregnated paper strips (Taxo *Haemophilus* Differentiation strips; BBL). Strips containing (i) haemin 4000 µg, (ii) NAD 100 µg, and (iii) haemin 2000 µg plus NAD 100 µg, were placed on plates of Mueller-Hinton Agar base inoculated with the organisms; *H. influenzae* was the control organism. Freshly isolated strains were always subcultured into chocolate-agar stabs or skimmed milk; these were kept at –70°C before shipment to Professor A.R. Ronald, University of Manitoba, Winnipeg, Canada, for further structural analysis.

Results

A total of 120 isolates of *H. ducreyi* was obtained. Very good growth of all the strains was observed on both media. They grew well at 34°C after incubation for 48 h. Growth was enhanced by CO₂ and a proportion of isolates, particularly on primary isolation, appeared to be CO₂ dependent. Increased humidity facilitated the growth of most strains. On most occasions a candle extinction jar containing a moistened filter paper incubated at 34°C provided an excellent environment for the culture of the organism. Growth was scanty after 24 h and the typical colonies were usually apparent only after 48–72 h. Colonies varied in size so that the variation in size and opacity often gave the impression of a mixed culture. Most colonies measured 2 mm in diameter. They were pinpoint, smooth, dome-shaped and buff-yellow to grey. The most remarkable feature was the extreme cohesiveness of the colony, allowing it to be nudged intact across the agar surface. It was not always easy to make a uniform suspension of the colonies in saline because the organisms stuck together in clumps. Microscopic examination of gram-stained films of colonies from solid media showed gram-negative coccobacilli in short chains, clumps or whorls, and at times in the typical rail-track arrangement. Bipolar staining could be observed in some.

All isolates were catalase-negative, and β-lactamase- and oxidase-positive. They produced neither indole nor H₂S. They required haemin (factor X) for growth but not NAD (factor V).

Discussion

In most North American and European STD clinics chancroid occurs too infrequently to warrant routine culture of genital ulcers for *H. ducreyi*. In epidemic settings such as the recent outbreak in California (Centers for Disease Control, 1982), culture for *H. ducreyi* has been performed on all patients with genital ulcers. In most tropical ende-

mic areas such as Kenya (Nzanze *et al.*, 1981; Piot *et al.*, 1983; Plummer *et al.*, 1983) improved techniques allow reliable isolation. Unfortunately, in developing countries laboratory resources cannot be made available to permit routine culture, but central laboratories should be able to undertake periodic surveys of genital ulcers to confirm the proportion due to *H. ducreyi* and to collect strains for determination of antimicrobial susceptibility patterns. Culture may also be required to establish a diagnosis in unusual or complicated patients.

Five thousand patients with probable chancroid are seen at the Nairobi City STC each year but only selected males with chancroid were included in the present study. Whilst use of heat-inactivated serum or clotted blood is no longer necessary because several agar media have been developed (Sottnek *et al.*, 1980; Bilgeri *et al.*, 1982; Nsanze *et al.*, 1984), it is possible that strain-specific variations of nutritional requirements occur. It is evident however, that all strains require a source of haemin and serum for optimal isolation. The addition of vancomycin makes media selective for *H. ducreyi*. An additional inhibitor such as polymyxin may be added (Plummer *et al.*, 1984). Penicillin or ampicillin would also be possible selective agents where, as in Nairobi, *H. ducreyi* produces β -lactamase (Fast *et al.*, 1982).

We recommend direct inoculation of media for luxuriant growth. No transport systems have been evaluated for maintaining *H. ducreyi* before primary isolation, but Amies' transport medium has been used with considerable success (Choudhary *et al.*, 1982).

The role of Gram's stain in the diagnosis of *H. ducreyi* infection in patients with genital ulcers remains controversial. Direct microscopic examination of smears from genital ulcers is not routine practice in this laboratory as, although few gram-negative organisms share the classical spatial distribution of *H. ducreyi*, in practice this characteristic appearance is rarely demonstrated in ulcer exudate (Nzanze *et al.*, 1981). Some authorities have found the evidence of gram-stained smears to be specific and sensitive (Taylor *et al.*, 1984), but others have

not (Chapel *et al.*, 1977; Nsanze *et al.*, 1981; Choudhary *et al.*, 1982).

The features of *H. ducreyi* on the media we describe are usually adequate for presumptive diagnosis in experienced hands. Additionally the typical rail track alignment of gram-stained organisms from culture plates can be helpful. The oxidase reaction depends upon the method used (Nobre 1982). It is uniformly positive if NNNN-tetramethyl-*p*-phenylenediamine is used as substrate but can be variable or negative when NN-dimethyl-*p*-phenylenediamine is used (Sottnek *et al.*, 1980). The catalase test has consistently been reported to be negative. However, Sturm and Zanen (1984) reported the tube test to be positive for all of their 29 isolates.

The porphyrin test is the preferred method for demonstrating the dependence of *H. ducreyi* strains on exogenous haemin (Hammond *et al.*, 1978*b*), but this requires special equipment and is not yet a routine procedure. This is also the case with the nitrate reductase and alkaline phosphatase tests (Sturm and Zanen, 1984). Moreover, tests for carbohydrate utilisation have been previously demonstrated to be negative (Kilian, 1976) and thus are not applicable in a busy laboratory. A comprehensive scheme for systematic identification of *H. ducreyi* has just been produced by Plummer *et al.* (1984). Such an algorithm will be very useful in larger regional STD reference laboratories which are well equipped and well stocked with reagents.

After primary isolation, most strains of *H. ducreyi* rapidly adapted to chocolate-agar media containing serum but without antibiotic. When subcultured in enriched chocolate agar stabs, organisms survived for up to 4 weeks. Strains could also be stored in serum or skimmed milk at -40°C to -70°C for longer periods. This survival enabled the transportation of *H. ducreyi* isolates from Nairobi to Winnipeg for further characterisation (McNicol and Ronald, 1984). These co-ordinated studies will determine whether different biotypes of *H. ducreyi* postulated by Sturm and Zanen (1984) do occur.

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