

Diagnostics

Diagnostic tests for chancroid

David A Lewis

Background

The identification of the causative agent of chancroid, *Haemophilus ducreyi*, was first reported in 1889 by August Ducrey, as a short streptobacillary rod with rounded ends, following experiments in which he autoinoculated patients' forearms with pus from their genital ulcers.¹ Bezancon *et al* subsequently inoculated the forearms of human volunteers with culture purified organisms and produced characteristic soft chancres from which the same organism was re-isolated.² This observation definitively identified *H ducreyi* as the causative organism of chancroid by fulfilling Koch's postulates.

Chancroid is a major cause of genital ulcer disease (GUD) in many resource poor countries of Africa, Asia, and Latin America although it remains relatively uncommon in the United States and Western Europe.³⁻⁴ Tender inguinal lymphadenopathy or bubo formation is a characteristic feature in up to 50% of chancroid patients.⁵ Genital ulcers may be caused by other sexually transmitted agents apart from *H ducreyi*, including *Treponema pallidum*, *Chlamydia trachomatis* serovars L1-L3, *Calymmatobacterium granulomatis*, and herpes simplex virus (HSV). It is therefore important to use appropriate diagnostic techniques in the management of patients presenting with the genital ulcer syndrome so that adequate treatment can be administered. In resource poor settings, where diagnostic facilities are not readily available, the World Health Organisation advocates the use of a syndromic management approach for the management of genital ulcer disease.⁶ Prospective and cross sectional case-control studies in Africa have provided substantial evidence that chancroid, either as a constituent of the GUD syndrome or as an aetiological diagnosis, is a risk factor for the heterosexual spread of human immunodeficiency virus (HIV).⁷⁻⁹ The few clinical studies published to date suggest that HIV seropositive men have increased numbers of genital ulcers which are slow to heal¹⁰⁻¹¹ and there are reports of increased therapeutic failures with single dose antimicrobial agents in HIV seropositive individuals.¹⁰⁻¹² There is, therefore, concern that the synergy between chancroid and HIV will further accelerate the HIV epidemic in those communities with a high prevalence of both diseases. Such concern has renewed research effort into developing better diagnostic techniques for chancroid and will be the focus of this review.

Reference standards in the assessment of diagnostic tests for chancroid

A major problem in the assessment of the diagnostic tests for chancroid in the pre-DNA

amplification era was the lack of a good reference standard for comparison. Older studies used either culture or clinical diagnosis of chancroid as "gold standards," both of which are now known to be inaccurate measures of the true incidence of chancroid in patients with genital ulcers. The accuracy of clinical diagnosis for *H ducreyi* infection appears to be related to both the prevalence of the chancroid in the population and to the experience of the attending physician in recognition of the disease. Single infections with *H ducreyi*, *T pallidum*, and HSV cannot be reliably distinguished by clinical presentation.¹³ Studies have shown that the accuracy of clinical diagnosis for chancroid appears to range from 33% to 80%.¹⁴⁻¹⁵ The increasing prevalence of HIV in chancroid endemic regions of the world makes it likely that this diagnostic accuracy will fall in the knowledge that previous HIV infection can modify both the appearance and clinical course of chancroid.¹⁰⁻¹¹⁻¹⁶⁻¹⁷ Variations in the ability to clinically diagnose chancroid accurately will obviously influence the calculated sensitivity for any diagnostic test being evaluated against this particular reference standard. DNA amplification technology now appears to be the most sensitive diagnostic method for chancroid and should be used as the reference standard in future evaluations of novel diagnostic techniques.

Diagnostic techniques for chancroid**MICROSCOPY**

H ducreyi is a Gram negative bacillus which exhibits an unusual tendency to autoagglutinate when growing in liquid culture or to form a cohesive colonial structure on agar plates. Microscopically, various morphological forms have been described such as "schools of fish," "railroad tracks," and "fingerprints." The organisms are visualised extracellularly more often than intracellularly and tend to occur in close proximity to polymorphonuclear leucocytes. Direct examination of clinical material by Gram's stain may be misleading because of the polymicrobial flora of most genital ulcers. Gram staining of clinical material does not compare favourably with either culture proved or clinically diagnosed chancroid cases in most studies (see table 1) and direct microscopy should not be used in the routine diagnosis of chancroid.¹⁸

IN VITRO CULTURE

In vitro culture for *H ducreyi* currently remains the main tool for the diagnosis of chancroid in the clinical setting and for many years was the "gold standard" for evaluating newer methods of diagnosis. However, the advent of more

Departments of
Genitourinary
Medicine and
Microbiology, Imperial
College School of
Medicine, St Mary's
Campus, Paddington,
London
D A Lewis
dalladal@aol.com

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Table 1 Range of sensitivity and specificity values for *H ducreyi* diagnostic tests

Diagnostic test	Sensitivity (%)	Specificity (%)	Reference standard	References
Microscopy	5–36	not stated	clinical diagnosis	56, 57
	10–63	51–99	culture	56, 58, 59
Culture	61–91	not stated	clinical diagnosis	27, 28
	35–75	94–100	PCR	19, 34, 37, 40
PCR	83–96	100	clinical diagnosis	36, 38
	56–100	52–100	culture	34, 38, 40
Antigen detection (IF)	93–100	63–74	culture	41, 42
	89	81	PCR	42
Non-adsorption EIA	55–89	49–96	culture	47
Adsorption EIA	81–100	23–88	culture	48
	53–83	57–71	PCR	51, 60
LOS EIA	83–96	97	culture	42, 49
	48–74	89–90	PCR	42, 51, 60

PCR = polymerase chain reaction, IF = immunofluorescence, EIA = enzyme immunoassay, LOS = lipo-oligosaccharide.

sensitive DNA amplification techniques has demonstrated that the sensitivity of *H ducreyi* culture is only about 75% at best.¹⁹ Lenglet has generally been given credit for the first successful in vitro culture of *H ducreyi* in 1898.²⁰ Teague and Deibert described successful culture of *H ducreyi* using fresh clotted rabbit blood heated to 55°C.²¹ Fresh clotted human blood²² and heat inactivated serum²³ from patients with clinically suspected chancroid have also been used as cultural methods in the past. Unfortunately, these methods were liable to contamination by other micro-organisms.²² Hammond *et al* addressed this problem by the selective culture of *H ducreyi* on vancomycin containing chocolate agar plates which had a sensitivity relative to clinical diagnosis of 44%.²⁴ It should be noted that inhibition of some clinical strains of *H ducreyi* by vancomycin (3 µg/ml) has since been reported.²⁵ Numerous selective artificial media have now been developed and have been reviewed elsewhere.²⁶

Nsanze *et al* evaluated the potential benefit of using more than one medium to isolate *H ducreyi* from clinical chancroid cases.²⁷ They reported that the rate of isolation and both the qualitative and quantitative growth of strains were best on gonococcal agar supplemented with 2% bovine haemoglobin and 5% fetal calf serum (GC-HgS) although some strains only grew on Mueller-Hinton agar supplemented with 5% chocolate horse blood (MH-HB). Both media contained vancomycin (3 µg/ml) and 1% CVA enrichment (Gibco Laboratories, Madison, WI, USA). In a study of 201 patients with clinical chancroid in Kenya, they determined that 71% of cultures were positive on GC-HgS and 61% were positive on MH-HB; the use of both media together increased the yield of positive cultures to 81%.²⁷ Dangor *et al* subsequently evaluated four different culture media in the primary isolation of *H ducreyi* from genital ulcer swabs of men with clinically diagnosed chancroid.²⁸ They reported that the combination of MH-HB and GC-HgS resulted in isolation of *H ducreyi* in 90% of cases; the use of all four media produced a marginally better isolation rate of 91%. Their study also demonstrated the potential of a simple inexpensive medium containing gonococcal agar base supplemented with 5% Fildes' extract and unchocolated horse blood as a suitable alternative

to GCHgS or MH-HB for diagnostic purposes in resource poor countries. In their hands, *H ducreyi* was isolated in 75% of cases on this medium. Another inexpensive medium containing 0.2% activated charcoal instead of fetal calf serum has also been described for use in resource poor countries.²⁹

As *H ducreyi* is a fastidious organism, it is essential that patients' specimens should either be plated out directly on an appropriate culture medium in the sexually transmitted disease (STD) clinic or sent to the microbiology laboratory for culture as soon as possible. There is no widely available transport medium although Dangor *et al* were able to retrieve viable *H ducreyi* after 4 days from specimens stored at 4°C in thioglycolate haemin based transport media.³⁰ Most *H ducreyi* strains grow best at 33°C in a water saturated atmosphere containing 5% carbon dioxide or in a traditional candle jar.³¹ It has been reported that better growth may be obtained using microaerophilic conditions in which *H ducreyi* inoculated culture plates are incubated in a closed anaerobic jar without a catalyst but with two carbon dioxide and hydrogen generating envelopes.³²

DNA AMPLIFICATION TECHNIQUES

In an attempt to improve the sensitivity of diagnosis, polymerase chain reaction (PCR) techniques have been developed.^{33–38} Primers for these PCRs have been designed to amplify sequences from either the *H ducreyi* 16S ribosomal RNA gene,^{33 36 37} the *rrs* (16S)-*rrl* (23S) ribosomal intergenic spacer region,³⁸ an anonymous fragment of cloned *H ducreyi* DNA,³⁴ or the *groEL* gene which encodes the *H ducreyi* GroEL heat shock protein.³⁵ The practical issues involved in *H ducreyi* detection by PCR have been reviewed recently.³⁹ A multiplex PCR (M-PCR) assay has also been developed for the simultaneous amplification of DNA targets from *H ducreyi*, *T pallidum*, and HSV types 1 and 2, and appears more sensitive than standard diagnostic tests for the detection of these aetiological agents in genital ulcer specimens.³⁷ The sensitivity of *H ducreyi* culture relative to M-PCR has been shown to be approximately 75% in two studies which sampled genital ulcers with swabs.^{19 37} Although PCR assays perform well on samples prepared from *H ducreyi* cultures, they appear to be less sensitive when used to test genital ulcer specimens owing to the presence of *Taq* polymerase inhibitors in the DNA preparations extracted from the specimens.³⁴ The sodium phosphate included in the specimen transport medium was postulated to be responsible for this observation.⁴⁰ The low specificity of PCR compared with culture in some of the above mentioned studies probably reflects the poor sensitivity of culture for diagnosing chancroid.^{33–35} This hypothesis is supported by observations that the use of additional confirmatory PCR assays, designed to amplify different DNA targets to the first PCR assay, were able to resolve discrepant PCR positive culture negative results.^{35 37} Virtually all the discrepant results were positive in the confirmatory PCR

based assays providing additional evidence that the sensitivity of culture is less than optimal.

ANTIGEN DETECTION

Karim *et al* tested a monoclonal antibody (MAb) raised against a 29 kDa outer membrane protein (OMP) in *H ducreyi* for its sensitivity and specificity as an immunofluorescence (IF) reagent using simulated vaginal smears containing *H ducreyi*, smears taken from skin lesions of mice infected with *H ducreyi*, and smears made from genital ulcer material and bubo aspirates from patients with clinically diagnosed chancroid.⁴¹ The IF test identified over 90% of culture positive cases of chancroid as well as some of the culture negative cases. The authors were unable to determine whether the IF positive but culture negative cases were in fact true positives as the more sensitive PCR based techniques were not available for confirmatory testing at that time. *H ducreyi* was detected in 95% of the mouse skin lesion smears using IF whereas the pathogen was only cultured successfully from lesion material in 14% of cases. The IF method also appeared to be more sensitive than culture in detecting *H ducreyi* in the simulated vaginal smears.

Ahmed *et al* assessed the diagnostic usefulness of a MAb (MAHD7) against *H ducreyi* lipo-oligosaccharide (LOS) in an indirect IF assay used to test genital ulcer (test group) or urethral (control group) smears in Zambian patients.⁴² By using PCR as the reference standard, the IF test was shown to have a sensitivity of 100% and a specificity of 74% in comparison with culture and a sensitivity of 89% and a specificity of 81% in comparison with the PCR assay. The authors reported that their MAb based IF assay was superior to bacterial culture which means that it may be a good candidate for use in diagnostic tests in high chancroid prevalence populations. Given that MAb MAHD7 used in this study was cross reactive with *H influenzae* strain 2019 and two strains of *Aeromonas hydrophila* on laboratory testing, it is debatable whether the IF test would perform as well in low chancroid prevalence populations but this remains to be determined. Immunofluorescence based techniques may not be suitable for resource poor countries, where *H ducreyi* infection tends to be most prevalent, because of the expense of purchasing and maintaining a fluorescent microscope.

Hansen *et al* designed an immunolimus assay which combined the specificity of a MAb raised against *H ducreyi* LOS with the sensitivity of the chromogenic *Limulus* amoebocyte lysate test for endotoxin.⁴³ This assay could detect purified *H ducreyi* LOS at a level of 25 pg/ml and could detect as few as 1000 colony forming units (CFU) of in vitro grown *H ducreyi* cells per ml of buffer. It appeared more sensitive than culture in detecting *H ducreyi* in lesion material obtained from infected rabbits.

NUCLEIC ACID PROBE TECHNOLOGY

H ducreyi DNA may be detected by the technique of DNA-DNA hybridisation using labelled *H ducreyi* derived probes. Parsons *et al*

evaluated the ability of three ³²P labelled DNA probes to hybridise with *H ducreyi* DNA in both bacterial suspensions and infected rabbit lesion material blotted onto nitrocellulose membranes.⁴⁴ The probes reliably detected 10⁴ CFU of *H ducreyi* in pure and mixed cultures. The sensitivity of this technique for diagnosis of chancroid would be greatly increased if initial amplification of *H ducreyi* DNA in the specimen could be made possible either by direct bacterial growth or by a DNA amplification based methodology. The design of probes that target single stranded rRNA molecules, present in large numbers in the bacterial cell, represents another approach to increase the sensitivity of oligonucleotide probe technology. Rossau *et al* chemically synthesised oligonucleotides complementary to different regions in the 16S and 23S rRNA molecules of *H ducreyi*. Experiments using these probes demonstrated DNA-RNA hybridisation to be a highly specific detection method for *H ducreyi* when testing culture grown isolates although no data were provided concerning the sensitivity of this technique.⁴⁵ There are no published data evaluating the usefulness of DNA or rRNA probe technology in the diagnosis of chancroid using clinical specimens as the source of *H ducreyi*.

SEROLOGICAL TESTS

Techniques used to detect serological responses to *H ducreyi* infection in humans and experimental animals include enzyme immunoassays (EIAs), dot immunobinding, agglutination, complement fixation, and precipitation.^{5 46-48} EIAs using ultrasonicated whole cell antigen,^{47 48} purified *H ducreyi* LOS⁴⁹ or OMPs⁴⁹ as antigens have been evaluated in serological diagnosis of chancroid. The presence of cross reacting antibodies to other *Haemophilus* species complicates the interpretation of serological testing results and it has been demonstrated that previous adsorption of sera with *H influenzae*,⁴⁸ *H parainfluenzae*,⁴⁸ and *H parahaemolyticus*⁵⁰ improves the specificity of the EIA. Using M-PCR as the gold standard for diagnosing *H ducreyi* infection in a group of patients with genital ulcers, Chen *et al* demonstrated that the adsorption EIA had a sensitivity of 53% and a specificity of 71% whereas the LOS EIA appeared less sensitive (48%) but more specific (89%).⁵¹ Both the sensitivity and the specificity of the adsorption EIA increased when follow up sera results were included in the analysis. These data suggest that the humoral response to chancroid develops as the disease progresses through the ulcerative stage. None of the sera from patients with experimentally produced *H ducreyi* pustules had significant levels of IgG antibodies to either LOS or ultrasonicated whole cell antigen when tested in the same study. This is in keeping with the lack of a humoral immune response to *H ducreyi* observed in the human experimental challenge model which is terminated at the pustular stage of infection with antimicrobial therapy.⁵²

Both *H ducreyi* OMP and LOS elicit primarily IgG responses that remain elevated for

several months which may explain observations that the adsorption EIA is less specific as a diagnostic tool in areas where chancroid is endemic.^{48, 49} Although serology based approaches to the diagnosis of chancroid may have limited sensitivity for the detection of circulating antibodies to *H ducreyi* in individual symptomatic patients, they may provide a useful tool with which to perform large scale epidemiological studies at the community level.

MASS SPECTROMETRIC METHODS

Matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI/TOF-MS) has been used with success to profile proteins from intact and disrupted bacteria. The MALDI/TOF mass spectra can be acquired in 10 minutes enabling identification of bacteria within a shorter time than is possible with conventional culture. Haag *et al* reported the rapid identification and speciation of *Haemophilus* bacteria using MALDI/TOF-MS and, in addition, used the technique to determine strain differences between different *H ducreyi* isolates.⁵³

BIOPSY

Characteristic histological features have been described in naturally acquired chancroid although tissue biopsy is not a recommended diagnostic method for chancroid.^{54, 55} Histological examination may be useful as a means to exclude malignancy in non-healing or atypical ulcers.

Final comments

At present, the only method available to most STD clinics for the diagnosis of chancroid is bacterial culture using specialised media designed to optimise the isolation of *H ducreyi* from clinical specimens. Although the use of more than one such medium increases the sensitivity of this technique, for most STD clinics this is not a practical option. In many cases, it may be necessary to ask the patient to return 24 hours later in order to allow sufficient time for the clinic's microbiology laboratory to prepare an appropriate culture medium. The main advantage of culture based over non-culture based diagnostic methods is that bacterial culture provides an isolate for antimicrobial sensitivity testing which may provide useful information in the event of treatment failure. With the nature and extent of antimicrobial resistance in *H ducreyi*, it is important to maintain some form of culture capacity, at least in reference centres. This would permit effective monitoring of antimicrobial resistance trends and aid in the management of suspected chancroid cases failing to respond to conventional therapeutic interventions.

In view of superior sensitivity of PCR based methods for the diagnosis of chancroid, it would be clearly advantageous for this technology to be available for routine diagnostic purposes as a replacement for, or an addition to, existing culture based methods. The M-PCR assay is a particularly attractive diagnostic tool in the investigation of patients

presenting with the GUD syndrome as it allows for the simultaneous detection of *H ducreyi*, *T pallidum*, and HSV types 1 and 2.³⁷ The M-PCR assay has been developed by Roche Products but is not yet commercially available.³⁹ MAb based technology also has the potential to provide a simple, inexpensive, rapid, and sensitive means of detecting *H ducreyi* in genital ulcer specimens but, again, no assay kits are commercially available at the present time. Serology has limited usefulness in the routine diagnosis of chancroid infection but may be useful in population based epidemiological research as a screening method for past infection.

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