

Laboratory techniques in the investigation of chancroid, lymphogranuloma venereum and donovanosis

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Introduction

Sexually transmitted diseases are not only highly prevalent in many populations of the developing world, but exhibit some special features. These include a high rate of complications and sequelae as a result of delayed or inadequate treatment, the important problem of antimicrobial resistance, particularly in *Neisseria gonorrhoeae* and *Haemophilus ducreyi*, and a high frequency of genital ulcer diseases.^{1,2}

We briefly review three causes of genital ulceration which are largely confined to the developing world, though all may be seen in Europe or North America as imported cases. In addition, outbreaks of chancroid have been reported from various western countries, and the disease has become even endemic in some populations of the USA.^{3,4} It therefore is not surprising that more research has been performed recently on chancroid than on donovanosis and lymphogranuloma venereum (LGV) which are rare conditions in the western world.

Some clinical and epidemiological features of tropical genital ulcers

Genital ulcerations are complex diseases and difficult to diagnose aetiologically on clinical grounds alone. Syphilis and genital herpes are common worldwide. The so called "tropical genital ulcer diseases" chancroid, lymphogranuloma venereum and donovanosis (granuloma inguinale) are infrequent in the industrialised world but cause major health problems in many developing countries. Interest in genital ulcerations has considerably increased following recognition that they enhance the risk of transmission of the human immunodeficiency virus (HIV) during sexual intercourse.⁵

Chancroid is caused by *Haemophilus ducreyi*. The disease starts with a painful papule at the site of infection resulting in a single or in multiple ulcers. Inguinal lymphadenopathy may be present in up to 50% of patients.

In patients with immunosuppression caused by HIV infection, extensive and persistent genital ulcers may be present without bubo development. Despite the fact that *H ducreyi* isolates of these patients show a normal antimicrobial sensitivity in vitro, the ulcers heal less frequently after short course treatment with cotrimoxazole or erythromycin, and often fail to respond to longer antimicrobial courses.

LGV is caused by *Chlamydia trachomatis*.

Three serovars L1, L2 and L3 are responsible for the vast majority of cases but other *C trachomatis* strains may also occasionally be isolated.^{6,7} LGV is a chronic disease with acute and late manifestations. A primary stage causing a small genital lesion is seen in a minority of patients. Most common is the secondary stage characterised by acute inguinal (and femoral) lymphadenitis with bubo formation. Late complications may occur in the anogenital area, such as ulcers, fistulas, strictures and elephantiasis.

Latent LGV may be reactivated in patients with HIV infection with development of multiple abscesses in the groins. The response to long courses of macrolides or tetracyclines is poor, and the destruction of inguinal lymph nodes often results in lymphoedema of the genitals, a chronic condition with persisting suppuration and pyoderma.

Donovanosis is a chronic infection of the genital region caused by *Calymatobacterium granulomatis*. The disease starts with a subcutaneous nodule at the site of infection. This nodule enlarges and erodes through the skin to reveal a red granulating ulcer. The disease may spread hematogenously resulting in cutaneous lesions at extragenital body sites.

Laboratory techniques

Chancroid The laboratory diagnosis of chancroid is based on the demonstration of the causative agent, *H ducreyi*. Direct examination of ulcer material on Gram-stained smears may contribute to the diagnosis if typical small Gram negative bacilli grouped in chains or "schools of fish" are observed. However, these typical features are infrequently seen on smears from patients with culture-proven chancroid resulting in a sensitivity of much less than 50%.⁸⁻¹⁰ In addition most genital ulcers harbour a polymicrobial flora due to secondary contamination and the presence of Gram negative bacilli in a smear may be misleading and frequently results in a false positive diagnosis.^{10,11} Thus, the specificity of a Gram stain for the diagnosis of chancroid is also less than 50%. Because of its low sensitivity and specificity, microscopic examination of a Gram-stained smear is not recommended for the diagnosis of chancroid.

Accurate diagnosis of chancroid depends on the ability to culture *H ducreyi*. Different isolation media have been used with varying success. For a review see table 1. Nutritional requirements of *H ducreyi* seem to be geographically defined and may partly explain the variation in detection rates of particular culture

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Table 1 Comparison of recovery rates on different culture media for isolation of *Haemophilus ducreyi*

Study (reference)	Site	Total number of isolates	Rate of isolation (%)
Nsanze 1984 ¹²	Nairobi	163	MH* : 75 GC ¹² : 88
Dylewski 1986 ¹³	Nairobi	75	MH* : 71 GC : 89
Kunimoto 1986 ¹⁴	Nairobi	27	MH* : 74 GC : 96
McDonald 1987 ¹⁵	Nairobi	57	Bieling ¹⁵ : 89 MH* : 53 GC : 72
Taylor 1984 ¹⁶	Bangkok	45	Sheffield ²⁰ : 2 MH† : 93
Dieng Sarr 1991 ¹⁷	Dakar	46	Heart Infusion ¹⁷ : 76 MH† : 100
Bogaerts 1989 ¹⁸	Kigali	38	GC : 4 MH† : 76
Bogaerts 1991 (unpublished)	Kigali	44	GC : 37 MH† : 82 GC : 36

MH* : Mueller Hinton enriched with chocolate horse blood and Isovitalex.

MH† : Mueller Hinton enriched with chocolate horse blood, Isovitalex and 5% foetal calf serum.

MH‡ : Mueller Hinton enriched with 1% hemoglobin, 1% VX supplement and 5% foetal calf serum.

media in different studies.¹² It has been shown that the parallel use of two media may increase the isolation rate of *H ducreyi* to above 80%, for cases with clinical chancroid; the two media can be incorporated into a single biplate to facilitate their use.^{12 13}

To obtain optimal growth, plates have to be incubated at 33–34°C in a 5–10% CO₂ and water saturated atmosphere for 3–4 days. Colonies of *H ducreyi* may vary in size depending on incubation temperature and atmosphere, growth medium and culture incubation time. Colonies are nonmuroid, raised and granular and have a grayish-yellow colour and can be pushed intact across the surface of the agar with an inoculating loop. Colonies are either translucent or opaque and this variability in opacity gives the impression of a mixed non pure culture. *H ducreyi* is a fastidious organism with limited biochemical activity. Haemin is required to initiate growth. Nitrate reduction and alkaline phosphatase are important characteristics. *H ducreyi* is oxidase positive when tested with tetramethyl-p-phenylene-diamine. Most isolates (nearly 100% in Africa) are β -lactamase positive.²² A presumptive identification of *H ducreyi* may be based on colony characteristics, Gram stain and eventually β -lactamase production and oxidase.^{21 22} Recently, chromosomal DNA probes and ribosomal RNA-derived oligonucleotide probes have been described which can be used for DNA hybridisation on bacterial cells to confirm the identification of *H ducreyi* in culture.^{23 24}

To determine the antimicrobial susceptibility of *H ducreyi*, agar dilution, broth microdilution and disk diffusion techniques have been used but there are no recommendations for a standard medium or standard method. Antibiotic susceptibility varies geographically. Resistance has been observed to sulfonamides, trimethoprim, penicillin, ampicillin, streptomycin, kanamycin, tetracycline and chloramphenicol. *H ducreyi* has remained susceptible to erythromycin and third generation cephalosporins. Table 2 summarises the antimicrobial susceptibility of

more than 1,000 strains of *H ducreyi* isolated in eight countries on four continents between 1978 and 1987, and also shows the mechanisms of resistance for sulfonamides, trimethoprim, tetracycline, streptomycin, kanamycin, chloramphenicol and ampicillin.

The use of non-culture techniques for diagnosing chancroid is very limited. Recently described dot immunobinding and enzyme immunoassays for the detection of circulating serum antibody show somewhat limited sensitivity and IgG assays do not differentiate between active and past infection and are currently more useful for epidemiology rather than for diagnosis.^{34 35} Geographical differences in the outer membrane profiles and antigenic composition of *H ducreyi* as well as the qualitative and quantitative differences in the human immune response to chancroid are important obstacles to the development of diagnostic immunoassays.

Monoclonal antibodies produced against outer membrane proteins and against lipopolysaccharide react specifically with *H ducreyi* by using an immunofluorescence technique³⁶ (and E. Roggen, unpublished). The performance of a monoclonal immunofluorescence assay directly on clinical specimens has proven to be competitive with culture in sensitivity but to have a specificity of 60% only.³⁶

DNA probes for the diagnosis of *H ducreyi* directly in clinical specimens have not been evaluated yet and the probes used for culture confirmation are radioactively labelled with ³²P, which is a handicap for routine use.^{23 24}

Lymphogranuloma venereum The laboratory diagnosis of LGV may be based on positive chlamydial serology, isolation of *C trachomatis* from the infected site, and histological identification of chlamydia in infected tissue. In the differential diagnosis between LGV and other possible conditions, syphilis and herpes should always be considered. In serology three types of techniques are used: the complement-fixation (CF) test, the single L-type immunofluorescence test and the micro-immunofluorescence test (micro-IF). In general, a fourfold rise of antibody in the course of suspected illness is diagnostic of active infection. However, seroconversion is demonstrated in only a minority of cases since most patients are seen by the physician after the acute stage. Moderate or high serum titres may also be caused by other *C trachomatis* infections and may persist for many years. Single-point CF titres of $\geq 1:64$ are seen in the majority of patients and are considered indicative for active LGV.³⁷ Single L-type chlamydial fluorescence seems to be more sensitive than CF, but also broadly cross-reacts with serovars of *C trachomatis* and, possibly, *C pneumoniae*, and thus shows the same inconvenience as CF.^{38 39} The most accurate diagnostic serologic assay is the micro-IF test. During the active phase of LGV patients usually show high levels of IgM (> 1:32) and IgG (> 1:512) with the antigen type of the infecting strain and much lower cross-reactivity with other *C trachomatis* strains.^{39 40}

Table 2 Antimicrobial susceptibility of clinical isolates of *H ducreyi*

Antimicrobial	MIC range (mg/l)*	Mechanism of resistance	Molecular mass
Sulfonamides	0.25-> 128	plasmid	4.9 MDa
Ampicillin	0.03-> 128	plasmid	3.2 MDa; 5.7 MDa; 7.0 MDa
Tetracycline	0.125-128	plasmid	30 MDa; 34 MDa
Kanamycin	0.5-128	plasmid	3.1 MDa
Chloramphenicol	0.25-16	plasmid	34 MDa
Trimethoprim	0.125-32	transposon on chromosome	
Erythromycin	0.0002-0.125	none	
Ciproflaxacin	0.002-0.125	none	
Ceftriaxone	0.001-0.06	none	

*Source: Hammond *et al.*²⁵ Sanson Le Pors *et al.*²⁶ Sloomans *et al.*²⁷ Bilgeri *et al.*²⁸ Sturm,²⁹ Taylor *et al.*³⁰ Bowmer *et al.*³¹ Dangor,³² Morse.³³

A major disadvantage of the micro-IF is that commercially ready to use reagents are not available and it therefore is primarily used in a few specialised reference laboratories. Cell culture is another method used for the diagnosis of LGV. *C trachomatis* can be isolated from bubo pus, genital ulcer and rectal tissue on cycloheximide treated McCoy cells or DEAE treated Hela 229 cells, but the recovery rate seems to be lower than 50%.⁴⁰ Material from the genital ulcer may be inoculated directly into cell tissue culture but bubo pus and tissues have to be homogenized in tissue culture medium to obtain a 10–20% (W/V) suspension, and 10⁻¹¹ and 10⁻² dilutions are inoculated into tissue culture. This is necessary to reduce a toxic effect of the pus on the culture cells.

Donovanosis The causative agent *Calymatobacterium granulomatis* appears in vacuoles in the cytoplasm of large histiocytes and occasionally in plasma cells and polymorphonuclear leukocytes. The intracellular organisms are called Donovan bodies and have a prominent clear capsule when mature. Culture of the bacterium in the chicken embryonic yolk sac has been reported, but is unsuccessful on artificial culture media.^{41 42}

The clinical manifestation is highly suggestive of granuloma inguinale and may be confirmed by Giemsa's, Leishman's or Wright's stain of a smear prepared from the lesion. A piece of clean granulation tissue is removed with a thin scalpel and crushed and spread on a slide and the impression obtained is air dried and stained. Deep coloured ovoid bacteria with or without capsule and with a closed safety-pin appearance are typical. Recently, a simple and more rapid diagnostic method has been described using a cotton swab for specimen collection and a one minute rapid differentiation staining technique, using eosin and thiazine dye solutions. As compared with a classic stain, no discrepant results were obtained.⁴³

Donovanosis is likely to be confused with a number of diseases affecting the genital region. Perianal lesions may simulate condylomata lata. Dark field microscopy and serology may exclude syphilis. Appropriate culture may help differentiate chancroid. Other diseases which should be excluded are tuberculosis, amoebiasis, schistosomiasis and carcinoma. Histological aspects of a biopsy specimen may be helpful: an ulcer with a mixed inflammatory infiltrate of plasma cells, neutrophils and histiocytes, with a conspicuous absence of lym-

phocytes suggests granuloma inguinale, and the use of Warthin-Starry silver impregnation stain demonstrating characteristic intracellular organisms (Donovan bodies) is diagnostic.⁴⁴

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